PhD Thesis

EFFECTS OF 3-NITROPROPIONIC ACID ON THE CORTICAL AND PERIPHERAL NERVOUS ACTIVITY IN RATS

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The Applicant's Relevant Publications

1. *Szabó A.*, Pecze L., Papp A. 2003. Effects of two different mitochondrial toxins on the spontaneous and evoked cortical activity in rats; Proceedings of the 10th Symposium on Analytical and Environmental Problems. SZAB, Szeged, pp.101-105.

2. *Szabó A.*, Pecze L., Papp A. 2004. Changes in the spontaneous and evoked cortical activity of rats induced by two mitochondrial toxins. Centr Europ J Occup Environ Med 10: 5-11.

3. Fazakas Z., *Szabó A.*, Lengyel Zs., Nagymajtényi L. 2005. Functional neurotoxic effecs to acute application of malonic acid in rats. Centr Europ J Occup Environ Med 11: 150-158.

4. *Szabó A.*, Fazakas Z., Papp A., Nagymajtényi L. 2005. Acute effects of two mitochondrial toxins, 3-nitropropionic acid and malonic acid, on the spontaneous and evoked cortical activity in rats. Centr Europ J Occup Environ Med 11: 144-150.

5. *Szabó A.*, Papp A., Nagymajtényi L. 2005. Functional neurotoxic effects in rats elicited by 3-nitropropionic acid in acute and subacute administration. Environ Toxicol Pharmacol 19: 811-817. Impact factor:1.253

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Summary

3-nitropropionic acid (3-NP) naturally present in some plants, intoxicating grazing farm animals, and is produced by food-infesting moulds, resulting in human poisoning by 3-NP. In human victims, the clinical signs included grimacing, sustained athetosis, chorea, torsion spasm, opisthotonos, hemiballismus and painful spasms of the extremities. CT revealed bilateral hypodensity, predominantly in the putamen with lesser involvement of the globus pallidus, and only occasionally of the caudate. Based on that 3-NP is frequently used in animal models of Huntington's disease (HD) as 3-NP induces striatal lesions resembling those seen in HD.

The primary mechanism of action of 3-NP is selective inhibition of the enzyme succinate dehydrogenase, which is a component of mitochondrial complex II, thus resulting in energetic insufficiency, which leads finally to excitotoxicity due to reduced ATP level, influx of Ca^{2+} via NMDA receptor activation, depression of the Na-K pump etc. Specific striatal lesions are explained by the local coincidence of glutamatergic excitotoxicity, dopaminerg toxicity, vulnerability of the lateral striatal artery and dysfunction of the blood-brain barrier. The mentioned effects themselves, however, are not restricted to the striatum or to the brain as a whole. It was thus likely that some other alterations in the function of the central and/or peripheral nervous system, detectable by electrophysiological methods, are also induced by 3-NP. In the literature, however, such reports were virtually missing.

In the present work, the well-established experimental methodology of the Department – recording of spontaneous and stimulus-evoked cortical, and evoked peripheral, nervous electrical activity in rats – was combined with intraperitoneal administration of 3-NP.

Adult male Wistar rats were used in the experiments. Four different timing schemes, *immediate, acute, subacute* and *subchronic. Immediate* effects were tested by anesthetizing and preparing the rats first. Central and peripheral electrical activity was recorded as pre-administration (self) control, then 3-NP (20 mg/kg b.m.) was given intraperitoneally and further records were taken for ca. 2 hours. For *acute* treatment, the animals received 3-NP in a single dose of 10 or 20 mg/kg b.m., i.p., 24 h prior to electrophysiological recording. In the *subacute* experiments, the rats were given 10 or 15 mg/kg b.m. 3-NP i.p. on 5 consecutive days. Then the rats were kept for 28 days before electrophysiological recording. In the

subchronic protocol, the 7 weeks old animals were administered 10 or 15 mg/kg b.m. 3-NP intraperitoneally on every fourth day, altogether six times. Electrophysiological measurements were done one week after the last injection.

The animals were prepared for electrophysiological recording in urethane anaesthesia. The left hemisphere was exposed removing the parietal bone. Wounds were sprayed with 10% lidocaine. The rat was put aside for at least 30 min for recovery. After that, ball-tipped silver recording electrodes were placed on the dura over the primary somatosensory projection area of the whisker pad (barrel field), and over the primary visual and auditory focus to record spontaneous (electrocorticogram, ECoG) and stimulus-evoked cortical activity (evoked potential, EP). In subacute experiments, one steel needle electrode was inserted in the caudato-putamen and the globus pallidus. Somatosensory (SS) stimulation was done by electric pulses delivered to the contralateral whisker pad. Visual (VIS) stimulation was performed by flashes directed into the contralateral eye. For auditory (AUD) stimulation, sound clicks were applied. Compound action potential of the tail nerve was recorded by means of a pair of stimulating needle electrodes inserted at the base of tail, and another pair for recording in a distance of 50 mm. One recording session consisted of six minutes recording of ECoG from the three sensory cortical areas simultaneously (and from the two subcortical nuclei). Then, EPs were recorded from the cortical areas via the same surface electrodes, and finally the compound action potential of the tail nerve. All stimulation, recording and subsequent analysis was controlled by the NEUROSYS 1.11 system (Experimetria Ltd., U.K.). The standard frequency of stimulation was 1 Hz. Double-pulse (supposed to be more sensitive to a toxic influence affecting energy supply) SS stimulation, with different interstimulus intervals (60 to 300 ms; repetition time, 1 s) was used in the immediate and acute treatment protocol, to investigate the dynamic interaction of successive excitation processes. The refractory period in the tail nerve was investigated with an interval of 10, 5, 3, 2 and 1 ms in all treatment schemes. Frequency of the evoked activity was determined 2 and 10 Hz somatosensory stimulation, and 20, 50 and 100 Hz tail nerve stimulation. After all recordings, the animals were sacrificed by an overdose of urethane.

From the ECoG records, the relative spectral power by the standard frequency bands, delta to gamma, was determined. The recorded evoked responses were averaged automatically, and their parameters were measured manually. On the EPs, onset latency and

onset-to-end duration was measured, on the SS EP, also peak-to-peak amplitude and peak latency. The measured parameters of the tail nerve action potential were onset latency and peak amplitude. From the double-pulse SS records, the second:first ratio for amplitude and latency was calculated, and from the tail nerve double-pulse records, relative and absolute refractory periods. Tail nerve conduction velocity was calculated from the onset latency and the distance of the electrodes.

In the spontaneous activity of the three cortical sites, there was a transient decrease in delta, and increase in beta2 and gamma activity, following immediate 3-NP administration, which then turned to the opposite with predominance of slow wave activity. In the acute model the SS ECoG changed dissimilarly in the two dose groups: increase in the fast, and decrease in the slow bands in the low-dose group, and the opposite change in the high-dose group. In the visual area, decrease in the slow bands was seen in both dose. In subacute application, the most pronounced ECoG alteration was the significant decrease in the theta activity, seen in all three cortical recording sites and in the caudato-putamen in the high dose, and in the SS and AUD area in the low dose animals. Generally, fast bands increased and slow bands decreased. The overall effect of subchronic 3-NP treatment on the ECoG was to reduce the slow band, and to increase fast band activity in all three cortical foci.

In immediate 3-NP application, the changes in the latency and duration of the EPs were not significant, even with varying the stimulation frequency. In case of double-pulse SS stimulation, the second:first ratio of the EP amplitude was around 0.5. After injection of 20 mg/kg 3-NP, the ratio started to increase, and considerably surpassed 1. This effect was, depending on interstimulus interval and time after 3-NP injection, significant. In acute 3-NP application, there was no noteworthy effect on the SS EP latency. The duration decreased, and this was significant at 100 ms stimulation period time. In contrast to the immediate effect, double-pulse stimulation failed to reveal significant differences between control and treated. There was clear increase in the latency of the VIS and AUD EP, and decrease in the duration of the latter. After subacute administration, increased latency of the sensory EPs was seen, significant in all recorded cortical areas and in both treated groups versus control. The alteration in the duration of the evoked responses was moderate. In subchronic application, duration of the SS EP increased, and the frequency dependence of this parameter was changed. In the other two modalities, both latency and duration decreased.

In the immediate and acute application, 3-NP caused no noteworthy changes in the conduction velocity and refractory period of the tail nerve. In the subacute scheme, conduction velocity increased and the relative refractory period decreased, but the frequency dependence of the amplitude indicated some damage. In subchronic application, an opposite, but not significant, change was seen in conduction velocity and relative refractory period.

Energy insufficiency caused by 3-NP results in a kind of tissue hypoxia. Exposure to low oxygen atmosphere caused, in volunteers, slowed EEG. In patients with inherited or idiopathic mitochondrial dysfunction, like mitochondrial encephalomyopathy (ME), abnormally slow EEG was observed. In some cases the connection of a given abnormal EEG pattern and the mutation of mitochondrial DNA could be verified, and electrophysiological methods were reported to be sensitive (although not specific) in detecting CNS disease due to mitochondrial abnormalities. Similar shift in the ECoG to low frequencies was observed in our study in the immediate, and partly the acute, 3-NP administration.

Among others, 3-NP affects glutamatergic transmission. Inhibited glutamate uptake contributes to excitotoxicity and leads to imbalance between excitation and inhibition. The resulting shortage of transmitter in the presynaptic ending may explain the significant decrease of somatosensory EP duration obtained by frequent stimulation in acute 3-NP treatment. Over a longer period, abnormally high level of glutamate is likely to desensitise receptors, expressed in the latency lengthening of the EP in all three modalities in the subacute treatment protocol. The glutamate receptors on the cholinergic neurons in the basal forebrain will also be first over-excited, then desensitized, with parallel alterations of the ascending cortical activation, contributing to the observed ECoG changes.

The results obtained by paired-pulse SS stimulation further support the adequacy of a disease model based on 3-NP and electrophysiological measurements. The change of the second:first ratio of the EP amplitudes following 3-NP administration reflects probably a kind of disinhibition. In human ME patients, paired-pulse somatosensory stimulation delivered to the median nerve revealed strongly reduced intracortical inhibition. The similarity of the effects, and the known connection of mitochondrial damage to increased cortical excitability suggest that double-pulse stimulation can be a sensitive and specific means to reveal the action of 3-NP in the CNS. In the acute protocol, no noteworthy effect on the second:first ratio was seen any more, indicating that the effect was probably directly caused by energy

insufficiency which is constantly present in ME patients but gradually developed and declined in the treated rats in the course of 24 hours.

The effect of 3-NP administration on the spectrum of ECoG was dissimilar or even opposite in different (most pronounced in subacute vs. subchronic) treatment protocols and high vs. low doses. This showed a good parallelism with the changes of motor behaviour described in the literature. The dependence of the extent and direction of the histological and behavioural outcomes on the dose has also been mentioned in the literature.

The mitochondrial effect of 3-NP is not restricted to the brain so the changes of the cortical electrical activity may have resulted from systemic mitochondrial damage. In our study no alteration in the body and organ weights were seen in the rats treated by the subacute and subchronic protocol on comparison with the untreated controls, so that such a secondary effect was unlikely. Evaluation of the alterations in the tail nerve parameters from this aspect showed that these were not in line with those of the cortical EPs, which again supports that the latter indicated an effect of 3-NP on the cortical activity itself.

The outcome of the present study showed that a functional approach, based on electrophysiological techniques, can be useful in detection and follow-up of the CNS effects of 3-NP (and possibly other agents used in modelling chronic degenerative diseases of the human brain) and can point to new questions. It can be concluded that the electrophysiological methodology established at the Department was suitable for detecting the functional changes caused by 3-NP. Further, the standard methods could be developed, primarily with the technique of repeated sensory stimulation, to accommodate it to the specific character of the alterations caused by 3-NP. The question, which of the parameters recorded and analysed is, based on sensitivity and specificity, best suitable to follow-up the development of damage caused by 3-NP, cannot be definitely answered at the moment. Is the necessary chronic recording technique available, SS stimulation with varied pulse pattern (double, slow-fast) seems promising. Another important question to be solved in the future is to what extent the functional changes indicated by the electrophysiolgical parameters and the histological or biochemical changes run in parallel. Answering these questions would provide another research tool, applicable in basic research and pharmacological development in the field of HD.

Table of Contents

Abbreviations

3-NP 3-nitropropionic acid

ampl. amplitude

AUD auditory

b.m. body mass

CNS central nervous sytem

CP caudato-putamen

DA dopamine

dur. duration

ECoG electrocorticogram

EEG electroencephalography

EP evoked potential

GABA γ-aminobutyric acid

GP globus pallidum

HD Huntington's disease

ISI interstimulus interval

lat. latency

ME mitochondrial encephalomyopathy

NMDA N-methyl-D-aspartate

SD standard deviation

SS somatosensory

VIS visual

1. Introduction

1.1. The extrapyramidal motor system. Basal ganglia. Huntington's disease

In the brain structure of humans and other mammals, two motor systems have traditionally been distinguished. One of them, pyramidal system, originates in the primary motor cortex (Brodman 4 and 6) and terminates directly in the spinal cord. In the other one, a number of subcortical structures are involved. This is called extrapyramidal system, although it is clear today that it is not a parallel, second system but has mutual connections with cortical and lower brainstem areas involved in motor functions.

The so-called basal ganglia constitute largely the extrapyramidal system. The basal ganglia comprise the striatum (consisting of the caudatum and the putamen, separate in humans but one structure in rodents), the globus pallidus or pallidum, the subthalamic nucleus and the substantia nigra. The crucial role of the basal ganglia in motor control was deduced, among others, from the central nervous system (CNS) diseases where a peculiar type of motor dysfunction could be matched with post mortem observed lesions.

Huntington's disease (HD), also called chorea maior, is one of such diseases. It is an autosomal dominant hereditary disease of slow progression. The symptoms typically appear around the age of 40 and the disease is fatal within 20 years. An earlier onset forecasts rapid progression. The victims are characterized by sudden, fast, twitch-like involuntary movements, their voluntary movements are, however, slowed. Beside the physical deterioration, dramatic mental changes also occur as well as dementia and psychiatric dysfunction (MacMillan and Quarrel, 1996). The disease has an average prevalence of 5 to 10 in 100,000, and has, as yet, no causal therapy. The most typical pathological sign in HD is the loss of neurons in the striatum, which is crucial also in the animal models of this disease. It turned out that a single gene and its product, the protein called huntingtin, is responsible for the disease, more exactly the abnormally long protein chain encoded by the mutated gene (Harper et al., 1996; MacDonald and Gusella, 1996). The whole brain strongly expresses huntingtin, and especially those neurons (medium spiny projection neurons) of the striatum where the histopathological damage is the most severe (Li et al., 1993; Ferrante et al., 1997). These GABAergic neurons project to the globus pallidus and are involved in the disinhibitory

mechanism of extrapyramidal motor control. Other types of neurons described in the striatum, the aspiny neurons, are interneurons (Alexi et al., 2000).

1.2. Animal models of Huntington's disease

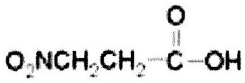
It has always been a major goal of research to develop animal models of human diseases. In case of Huntington's disease (HD) models, the primary aim was to induce striatal lesions resembling or identical to those seen in humans, and to see if the functional disorders will be like, too.

The neurochemically induced striatal lesions are based on the special characteristics of this area. Due to the corticospinal glutamatergic afferentation, there are many excitatory amino acid receptors in this nucleus. Glutamatergic transmission is known to be "risky" in terms of excitotoxic damage, first of all via N-methyl-D-aspartate (NMDA) receptors. Excitotoxins like quisqualic acid, quinolinic acid, iboteinic acid or kainic acid acting on these receptors when injected locally, provide a good model of the histopathology and the changes in receptors seen in HD. The dopaminergic innervation of the striatum from the substantia nigra constantly releases dopamine (DA) which can be neurotoxic, directly by increasing intracellular Ca^{2+} level, and indirectly by increasing glutamate release and by forming autooxidation products (Alexi et al., 2000). Finally, the mitochondria in the GABAergic medium spiny neurons of the striatum are extremely vulnerable. These three features are exploited together in the models based on mitochondrial toxins, the most important of them being 3-nitropropionic acid (3-NP). Damage obtained with 3-NP results in a pathology and symptomatology resembling to what is seen in HD (Borlongan et al., 1997b).

1.3. 3-nitropropionic acid

1.3.1. Identity and natural occurrence

The substance called 3-nitropropionic acid (synonyms: β-nitropropionic acid, bovinocidin, hiptagenic acid) is, in chemically pure form, a yellow, crystalline solid, readily soluble in water.



At physiological pH, 3-NP easily degrades, resulting in short half life of the substance after administration. In rats and mice, most of the 3-NP injected will be broken down within 40 minutes (Pass et al., 1994; Schulz et al., 1996).

There is a number of natural sources of 3-NP, which was a precondition to the cases of accidental poisoning, leading finally to the use of the substance in disease modelling. It is found in at least four higher plant families: Malpighiaceae, Corynocarpaceae, Violaceae and Leguminosae. Several economically important forage plants either contain glicosides of 3-NP itself, or those of its precursor, 3-nitropropanol (itself a succinate dehydrogenase inhibitor, and converted to 3-NP by alcohol dehydrogenase) (Pass et al., 1994). Such plants, belonging to the *Coronilla, Astragalus* and *Indigofera* genera of the Leguminosae family, cause poisoning of livestock mostly in western states of North America.

A unique source of naturally occurring 3-NP is the secretion of chrysomelide beetle, which is a chemical defence against predators (Pasteels et al., 1989).

Human 3-NP poisoning normally results from the production of the toxicant by moulds, namely *Aspergillus* and *Arthrinium* species. Although these can infest various foodstuffs, the typical sources of poisoning are sugarcane (consumed in China as a sweet delicacy) besides, fermented soybean paste and mould-infested commonly eaten foods, such as cheese, peanut or banana.

1.3.2. Poisoning by 3-NP in animals and humans: symptoms

In grazing animals, acute poisoning is characterised by incoordination, laboured breathing, cyanosis and weakness. In chronic poisoning, weight loss and poorly reversible hind limb ataxia was described (Pass et al., 1994).

Accidental human food poisonings with 3-NP used to be first widespread in China, named as "deteriorated sugarcane poisoning" or later as "mouldy sugarcane poisoning" caused by fungi growing on sugarcane stored under damp condition over winter. It was seasonally occurring during February-April, with ca. 10 % lethality and many cases of lifelong disability. The main epidemiological feature was the small number of persons in one outbreak, with the victims being mostly children and young people. (The reason is that sugarcane was supplied to children as a fresh fruit substitute in spring, when fruit prices were high and especially as a seasonal or holiday confectionery, and not that children would be more susceptible to 3-NP toxicity, as described in the next chapter). Generally, the incubation period was from 10 min to 8 h (mostly 2-3 h). Clinical signs included gastrointestinal irritation within 2-3 hours after ingestion (sudden-onset nausea, vomiting, abdominal pain and diarrhoea), which in mild cases progressed only to headache. Both spinal and cranial nerves were involved, with deviations of gaze and nystagmus being prominent features. Some patients also developed convulsions, decerebrate rigidity and coma. In such severe cases, convulsions and coma developed by 3-18 hours. The patients that regained consciousness were mute and incontinent. Some developed delayed dystonia 7-40 days later. Clinical signs were grimacing, sustained athetosis of hands and fingers, chorea, torsion spasm, opisthotonus, spasmodic torticollis, hemiballismus and painful spasms of the extremities. Computerized tomography of the poisoned patients revealed bilateral hypodensity, predominantly in the putamen with lesser involvement of the globus pallidus, and only occasional involvement of the caudate or claustrum (Ludolph et al., 1991; Fu et al., 1995) – which fact was of primary importance in developing the disease models. Abnormal electroencephalographic results were also seen, but body temperature, heart, liver, lung, cerebrospinal fluid, blood, urine and faeces were all normal. In adults, 3-NP caused gastrointestinal symptoms, whereas signs of severe encephalopathy were not common (such age dependence of severity was also observed in the inherited HD).

1.3.3. Toxicity and mechanism of action of the 3-nitropropionic acid

Experimental studies are done in rodents and in non-human primates. Oral or intraperitoneal LD_{50} doses of 3-NP in rats are in the range of 60-80 mg/kg. Subcutaneous LD_{50} is ca. 50 % lower (Pass et al., 1985; Burdock et al., 2001).

In rats, the progression of intoxication can be roughly divided into three stages characterized by somnolence in Stage I, uncoordinated gait with stereotypical paddling and rolling movements in Stage II and ventral or lateral recumbency with prominent chewing movements in Stage III. Length of time to a specific stage was inversely proportional to dose. Death usually results from respiratory failure (Hamilton and Gould, 1987). Severe motor abnormalities: dystonic posturing with hindlimb extension, wobbling gait; are most frequently seen after subacute administration, while in chronic treatment these are barely detectable (Guyot et al., 1997).

The principal mechanism of action is succinate dehydrogenase inhibition. 3-NP is a close structural analogue of succinic acid and selectively inhibits succinic acid dehydrogenase, which is a component of mitochondrial complex II and responsible for the dehydrogenation of succinic to fumaric acid in the Krebs cycle. The reaction of 3-NP with succinic dehydrogenase results in the production of 3-nitroacrylate, which then forms a covalent adduct with the enzyme's active site, leading to irreversible catalytic inactivation at a 1:1 molar ratio (Alston et al., 1977). Catalytic activity only recovers with de novo synthesis of the enzyme. Inhibition of succinic dehydrogenase therefore deprives the cell of one of the two major pathways for the generation of energetic and reductive intermediates. Malonic acid is another, reversible inhibitor of succinate dehydrogenase. Local injection of malonate into the striatum produces very similar lesions to that produced by 3-NP (Beal et al., 1994), further supporting that inhibition of succinate dehydrogenase is the primary mechanism of 3-NP toxicity.

The secondary mechanisms of action are not unique to 3-NP toxicity. Reversible systemic vascular damage, that is, a vasodilator reaction and reduced blood pressure due to NO generation in the breakdown of 3-NP, has been described (Castillo et al., 1993). A vascular explanation for vulnerability of the striatum to selective toxicity (hypoxia, ischaemic damage) was deduced from the unusual branching pattern of the striatal arterial blood supply

(Nishino et al., 1998). Inhibition of mitochondrial β-oxidation of fatty acids, caused indirectly by 3-NP, provides substrate for free radical formation leading to oxidative stress (Binenda and Kim, 1997; Esfandiari et al., 1997). Excitotoxicity of 3-NP is a consequence of impaired mitochondrial respiration resulting in reduced level of ATP and oxygen consumption (Erecinska and Nelson, 1994). Enzyme inhibition to 50 % was sufficient to trigger the excitotoxic cascade, namely, reduced ATP level resulted in decreased membrane potential leading to massive influx of Ca^{2+} via NMDA receptor activation, ultimately leading to apoptotis or necrosis (Coyle and Puttfarcken, 1993; Portera-Caillau et al., 1995). Apoptotic death is involved in neuronal loss produced by 3-NP, in the late stage, after the stage of recumbency. Depression of the Na-K pump due to metabolic disruption induced by 3-NP also leads to irreversible depolarisation, confirming the role of excitotoxicity in 3-NP pathogenesis (Riepe et al., 1992). Excitotoxicity secondary to metabolic deprivation would seem an excellent way of explaining selectivity, but areas such as the hippocampus which are vulnerable to classic acute excitotoxicity are not especially vulnerable to 3-NP. The combination of vascular involvement with the dopaminergic hypothesis (described in 1.2.) seems an efficient way of explaining the brain regional selectivity. The pattern of D2 receptor localisation in the brain shows remarkable similarities to the 3-NP lesion distribution (Nishino et al., 1997), with striatum being the most vulnerable site, and hippocampus, thalamus, and cerebral cortex only being involved in more severe cases. Striatum specific lesions are supposed to be due to glutamatergic excitotoxicity, dopaminerg toxicity, vulnerability of the lateral striatal artery, dysfunction of the blood-brain barrier and high activity in the glutamate transporter. In subchronic dose models irreversible loss of neurons in the striatum and, to a lesser extent, in the hippocampus, but not in the brainstem or spinal cord was seen (Beal et al., 1993).

Age and gender are further factors to influence the vulnerability of rats to 3-NP. Younger animals are less affected (Bossi et al., 1993), possibly because succinic dehydrogenase activity, and mitochondrial function generally, declines with age (Fattoretti et al., 1998) and males are more susceptible, which is in relation in female sex hormones (Nishino et al., 1998). Vulnerability to 3-NP may depend on genetic factors as strain differences was found (Ouary et al., 2000). In specific studies it was found that 3-NP is not

carcinogenic, does not cause reproductive toxicity (NCI, 1978), is not mutagenic (Oshiro et al., 1991) and only weakly genotoxic (Batiste-Alentorn et al., 1995).

1.4. The cortical and peripheral sensory systems of the rat involved in the experiments

In the experiments described hereunder, the somatosensory visual and auditory systems of the rat were used to take measurements.

Most of the records were made from the somatosensory (SS) cortex, more precisely from the projecton area of the whiskers (Par1; Zilles, 1984), which in case of rats are very important sensory organs. The follicles of the whiskers (vibrissae) are innervated by the intraorbital branch of the trigeminal nerve. The central axons project to the principal nucleus in the trigeminal complex of the brainstem, which sends projections to the medial ventral posterior nucleus of the thalamus. Thalamocortical projection terminates a special region of the somatosensory cortex, characterised by peculiar cytoarchitecture and called "barrel field" (Woolsey and Van der Loos, 1970).

Visual (VIS) information is conveyed from the retina by the axons of the retinal ganglion cells, via the optic nerve and optic tract, to the lateral geniculate nucleus of the thalamus. From this relay station the optic radiation conducts the action potentials to the ipsilateral primary visual cortex (Oc1B; Zilles, 1984). A parallel path comprises optic tract projections to the superior colliculus, responsible more for movement than for pattern detection, and connected to various subcortical targets.

In the auditory (AUD) pathway, the excitation of the cochlear hair cells is transferred by the auditory nerve to the cochlear nucleus. This in turn projects – both contra- and ipsilaterally – to the other auditory nuclei of the brainstem: the superior olivary complex and inferior colliculus. Auditory information, then, goes through the pathways of the medial geniculate body of the thalamus and projects to the primary and secondary auditory fields of the cortex (Te1; Zilles, 1984).

The tail nerve of the rat is a suitable substrate for examination of reactions of the peripheral nerves. There is a pair of dorsolateral and ventrolateral mixed nerve bundles in the tail. In its compound action potential, the spike of the motor and fast sensory fibres predominates.

1.5. Aims of the study

In this study, the well-established experimental methodology of the Department – recording of spontaneous and stimulus-evoked cortical, and evoked peripheral, nervous electrical activity in rats – was combined with a new type of neurotoxicant. The substance, 3-NP, has been used in animal models of HD, and the pathological and motor abnormalities caused have been described. There has been, however, hardly any report on the electrophysiological effects of 3-NP, although such an effect was, considering, among others, the universal mitochondrial damage induced by 3-NP, likely.

The first aim was, consequently, to see to what extent the mentioned methodology - that is, recording and analysis of the spontaneous activity of the somatosensory, visual and auditory cortical area, the sensory evoked responses from the same sites, and the compound action potential of the tail nerve, of anesthetised rats – was suitable for detecting the functional changes caused by 3-NP in different dosage schemes ranging from acute to subchronic. It was also a question how this method can be altered or broadened to better detect the effects. The final aim was to find a parameter which was sensitive to 3-NP. It was supposed that the results obtained may be a useful addition to the existing disease model, e.g. in following-up the appearance of the brain damage or in pharmacological research.

2. Materials and Methods

2.1. Experimental animals, housing and chemicals

Adult male Wistar rats, obtained at the Breeding Centre of the University, were used in the experiments. Depending on the actual experimental protocol (see 2.2.) animals of different age and body mass were used. The animals were housed under standard conditions (22–24 °C, 12 h light/dark cycle with light starting at 6:00 a.m., up to four rats in one cage) with free access to conventional standard rodent chow and drinking water.

3-NP, analytical grade, was obtained from Sigma-Aldrich GmbH, Steinheim, Germany. For administration, 3-NP was dissolved in distilled water to ca. 1.0 ml/kg b.m. administration volume. The correction of pH, mentioned by several authors, was omitted because there are literature data on the instability of 3-NP at near-neutral pH (degrading to nitrite resulted in loss of 3-NP) (Erecinska and Nelson, 1994; Ray, 1999) and because the discomfort of the animals during and after the intraperitoneal injection did not seem to be higher than in other experiments.

Urethane, used for anesthesia, was purchased from Reanal, Budapest.

2.2. Experimental protocols

Experiments were done in four different timing schemes, that is, immediate, acute, subacute and subchronic treatments were applied. These treatment schemes were chosen on the basis of literature as the most frequently used dosage and treatment time (Beal et al., 1993; McCracken et al., 2001) and as exposure models. Acute single or subacute dosage is used to model human exposure pattern of 3-NP, whereas repeated or continuous lower level dosage model is a pattern more common in grazing animals. Repeated dose protocols enable better survival, while acute dose models facilitate time course investigation of pathogenesis. On the other hand, the applied schemes roughly correspond with HD models found in the literature. All treatment and control groups consisted of 10 animals. For studying the effects of immediate and acute administration of 3-NP, rats of ca. 350 g body mass (10 weeks of age) were used. In case of subacute and subchronic treatment, younger rats (7 weeks old) of lower

body weight (ca. 180-190 g) were taken so that they did not grow over-aged and/or overweighted at the time of preparation.

Immediate effects were tested by anesthetizing and preparing the rats first (see 2.3.). The forms of central and peripheral electrical activity to be investigated were recorded several times for a pre-administration control, then 3-NP (20 mg/kg b.m.) was given intraperitoneally and further 6-8 records were taken.

For *acute* treatment, the animals received 3-NP in a single dose of 10 or 20 mg/kg b.m., i.p., 24 h prior to electrophysiological recording.

Effects of 3-NP, possibly developing over a longer period of time, were investigated in two protocols, designated here a *subacute* and *subchronic*. In the subacute experiments, the rats were given 10 or 15 mg/kg b.m. 3-NP i.p. on 5 consecutive days. Then the rats were kept for 28 days before electrophysiological recording. In the subchronic protocol, the 7 weeks old animals were administered 10 or 15 mg/kg b.m. 3-NP intraperitoneally on every fourth day, altogether six times. Electrophysiological measurements were done one week after the last injection. (Using the terms *subacute* and *subchronic* may be not fully correct in toxicological terms. They were retained, all the same, to stress the difference in the length of exposure periods.)

The immediate effect protocol was essentially self-controlled. All the same, parallel control rats, receiving an injection of distilled water, were also used. In the other protocols, a control group of rats (untreated but undergoing the same procedure) was always used, and the evaluation was based on the comparison of group averages.

2.3. Preparation of the animals

The animals were anaesthetized by inpraperitoneal injection of 1000 mg/kg b.m.urethane (Bowman and Rand, 1980). The head of the rats was clamped in a stereotaxic frame, the bony scull was exposed by a mid-sagittal cut through the head skin and removing the muscles and connective tissues adhering to the skull. Finally the left hemisphere was exposed by drilling along the inner circumference of the temporal bone by means of a mini drill, and removing the bone. Wounds were sprayed with 10 % lidocaine and the exposed cortex was covered with warm liquid paraffin. The rat, wrapped in a warm cloth, was put

aside for at least 30 min for recovery. After that, the rat was laid into the stereotaxic instrument of the electrophysiological apparatus. A thermostated (+36.5 °C) base plate secured the animal's normal body temperature during the recording procedure. To record spontaneous and evoked cortical activity, ball-tipped silver recording electrodes were placed on the dura over the primary somatosensory projection area of the whisker pad (barrel field), and over the primary visual and auditory focus. These sites were determined on the base of a somatotopic map (Zilles, 1984). A stainless steel clamp was attached to the cut skin as an indifferent electrode. In certain subacute experiments, one steel needle electrode was inserted in the caudato-putamen (stereotaxic coordinates: AP 0, L 3, V 5; Paxinos and Watson, 1982) and the globus pallidus (AP 2, L 3, V 6, Paxinos and Watson, 1982) to see if any alteration can be seen in the striatum – the principal site of action of 3-NP - by this investigation method. SS stimulation was done by a pair of needles inserted into the whiskery part of the nasal skin, delivering square electric pulses (see 2.4. for stimulation parameters). VIS stimulation was performed by flashes delivered by a flash generator via an optical fiber conductor directed into the contralateral eye of the rat. For acoustic stimulation, sound clicks were applied into the ear of the rat. Evoked activity of the tail nerve was taken by means of a pair of stimulating needle electrodes inserted at the base of tail (delivering like electric stimuli as used to stimulate the whiskers), and the compound action potentials were recorded distally by another pair of needles in a distance of 50 mm.

2.4. Electrophysiological investigation

One recording session consisted of six minutes recording of spontaneous activity (electrocorticogram, ECoG) from the three sensory cortical areas simultaneously (and then from the two subcortical nuclei in some subacute experiments. Then, evoked potentials (EP) were recorded from the cortical areas via the same surface electrodes, and finally the compound action potential of the tail nerve. The recorded biological signals were amplified (10^4x) and fed into the digitizer interface of the recording setup. Sensory stimuli were delivered by a digital time base and stimulator unit (Experimetria Ltd., UK). All stimuli were of just supramaximal strength (meaning that, e.g., the stimulus voltage was increased until the evoked response reached maximal amplitude and ca. 5% was added) and well above

2.5. Measurements and evaluations of the records

From the ECoG records, the relative spectral power by frequency bands: delta, 0.5-4 Hz; theta, 4-7 Hz; alpha, 8-13 Hz; beta1, 13-20 Hz; beta2, 20-30 Hz; gamma, 30-50 Hz (Kandel and Schwartz, 1985) was determined by the NEUROSYS software.

The recorded evoked responses were averaged automatically, and their parameters were measured manually with the help of screen cursors of the software. On the somatosensory EP, first of all onset latency was measured, between the stimulus artefact (designated "*0*" in Fig. 1) and onset of the first peak (*"A"* in Fig. 1). Duration of the EP was calculated as the difference of the *0-D* and *0-A* times. Where the amplitude of the EP was also of interest, the positive and negative peaks (*"B"* and *"C"* in Fig. 1) were considered, peak-to-peak amplitude being measured between *B* and *C* (along the Y axis) and peak latencies between *0* and *B* or *C*. In case of the visual and auditory EPs, only onset latency and duration was measured, in an analogous way. The tail nerve action potential had also a biphasic shape. On this form of activity, onset latency was defined analogously with the *0-A* distance, and the peak-to-peak amplitude was analogous with the *B-C* difference.

In the cortical double-pulse SS records, the second:first ratio for amplitude and latency was calculated, and in the tail nerve double-pulse records, relative and absolute refractory periods (as described by Dési and Nagymajtényi, 1999 and Anda et al.,1984). Tail nerve conduction velocity was calculated from the onset latency and the distance of the electrodes, according to Miyoshi and Goto (1973) with the modification that recording was made at room temperature.

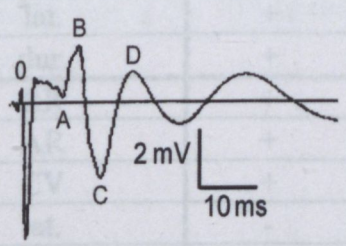


Figure 1 Typical example of the somatosensory evoked potential with the specific measuring points. See text for details.

2.6. Statistical analysis of the data

The results of subchronic, subacute and acute exposure were tested for significance with one-way ANOVA. For the results of the immediate treatment (pre- and post-application data), repeated measure ANOVA was used. Post hoc analysis was done by LSD with $p<0.05$ as limit.

Table 1 shows what parameters were measured in each treatment scheme.

Table 1 Summary of the measurements made in each treatment scheme.

	site	type of impulse	measured parameters	immediate treatment	acute treatment	subacute treatment	subchronic treatment
spontaneous activity	SS	-	-	+	+	+	+
	VIS	-	-	+	+	+	+
	AUD	-	-	+	+	+	+
	CP	-	-	-	-	+	-
	GP	-	-	-	-	+	-
evoked activity	SS area	single	lat.	+	+	+	+
			dur.	+	+	+	+
		double	lat.	+	+	-	-
			ampl.	+	+	-	-
	VIS area	single	lat.	+	+	+	+
			dur.	+	+	+	+
	AUD area	single	lat.	+	+	+	+
			dur.	+	+	+	+
	tail nerve	double	RR	+	+	+	+
			AR	+	+	+	+
			CV	+	+	+	+
		single	lat.	-	+	+	+
			ampl.	-	+	+	+

3. Results

3.1. Immediate effects of 3-NP

3.1.1. Effects on the spontaneous activity

In the immediate exposure model, the spontaneous activity of the SS, VIS and AUD areas showed a uniform trend of alteration within ca. 2h after administration of 20 mg/kg b.m.3-NP (Fig. 2A, B, C). 10 minutes after administration, delta activity decreased, whereas beta2 and gamma activity increased. 30 minutes after the administration, these changes returned to the control level, but around the 60th minute, an opposite shift appeared (increase in delta, theta and alpha activity, and decrease in beta1, beta2 and gamma activity), compared to the pre-administration control. By the 100th minute after administration, this change - predominance of slow wave activity - became more and more expressed, but remained below the level of significance.

3.1.2. Effects on the somatosensory evoked potential

Up to the 30th minute after the administration of 3-NP, latency of the somatosensory EP, obtained with 1000 ms stimulation period time, decreased. From then on, it started to increase until the end of the recording, however did not go over the pre-administration control level, and no significance was observed (Fig. 3A). The same trend was seen in the alteration of duration evoked with 1000 ms period time, the only difference was that the deepest point of the slight decrease of the parameter was at the 10th minute, and by the end of the recording it almost reached control level (Fig 3B).

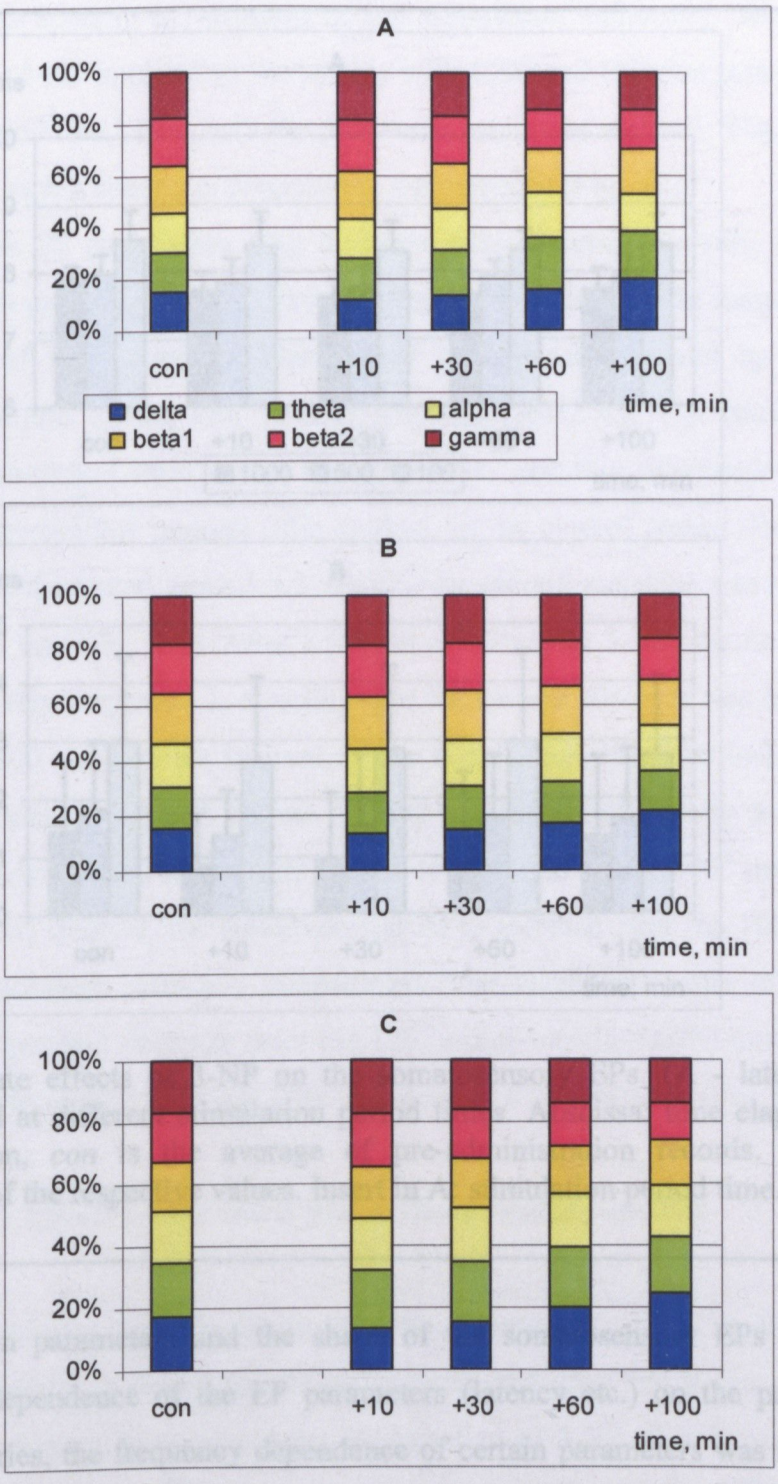
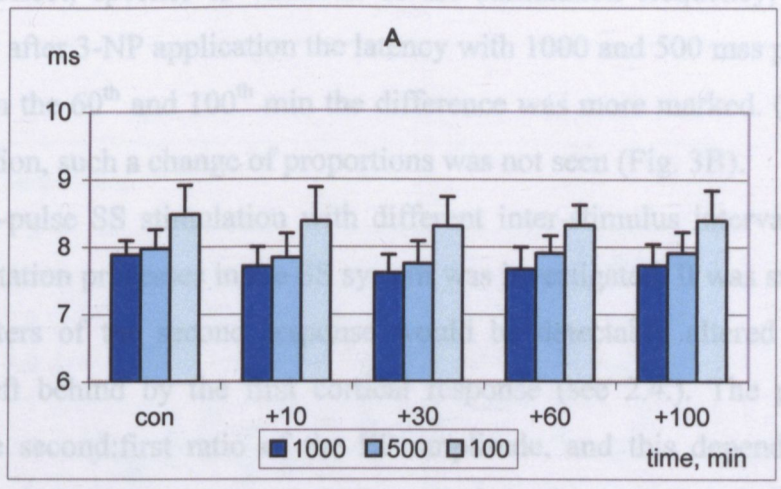


Figure 2 Effects of 3-NP on the frequency spectrum of the spontaneous cortical activity (A - somatosensory; B - visual; C - auditory area) in immediate administration. Abscissa: time elapsed after drug administration, *con* is the average of pre-administration records. Ordinate: relative power of the standard frequency bands (means, n=10). Insert in A: bar pattern of the frequency bands.



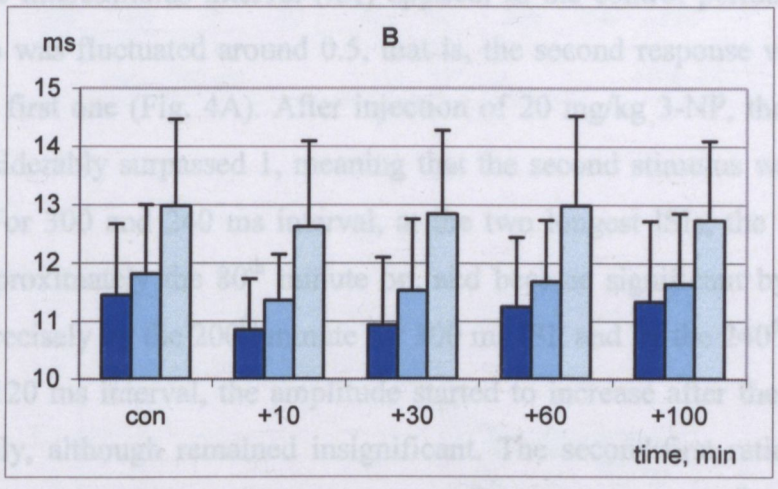


Figure 3 Immediate effects of 3-NP on the somatosensory EPs. (A - latency; B - duration), obtained at different stimulation period times. Abscissa: time elapsed after drug administration, *con* is the average of pre-administration records. Ordinate: mean+SD (n=10) of the respective values. Insert in A: stimulation period time.

The stimulation parameters and the shape of the somatosensory EPs allowed the investigation of the dependence of the EP parameters (latency etc.) on the parameters of stimulation. In one series, the frequency dependence of certain parameters was investigated, based on the supposition that these can be altered by the diminishing mitochondrial energy supply in neurons affected by 3-NP (see 2.4.). Thus, the train of 50 stimuli was applied, beside 1000, with 500 and 100 ms period time. The latency values obtained by the more frequent stimulation showed the same slight alterations after application of 3-NP, as was seen with the standard, slow stimulation.

The only effect, specific to variation of the stimulation frequency, was that while before and shortly after 3-NP application the latency with 1000 and 500 mss period time were hardly different, in the 60th and 100th min the difference was more marked. (Fig. 3A). In the values of EP duration, such a change of proportions was not seen (Fig. 3B).

By double-pulse SS stimulation with different inter-stimulus intervals, the dynamic interaction of excitation processes in the SS system was investigated. It was supposed that the measured parameters of the second response would be detectably altered by the relative refractory state left behind by the first cortical response (see 2.4.). The parameter to be evaluated was the second:first ratio of the EP amplitude, and this depended on the time elapsed and on the interstimulus interval (ISI) applied. In the control period (from –60 to 0 minutes), the ratio was fluctuated around 0.5, that is, the second response was considerably suppressed by the first one (Fig. 4A). After injection of 20 mg/kg 3-NP, the ratio started to increase, and considerably surpassed 1, meaning that the second stimulus was less inhibited by the first one. For 300 and 240 ms interval, at the two longest ISIs, the increasing trend appeared from approximately the 80th minute on, and became significant by the end of the recording; more precisely by the 200th minute for 300 ms ISI, and by the 240th minute for 240 ms. For 180 and 120 ms interval, the amplitude started to increase after the 180th and 210th minute, respectively, although remained insignificant. The second:first ratio of the latency showed unitary, ISI-dependent decrease from the 120th minute to the 160th minute, and after that it did not change. No significance was seen in change of this parameter (Fig. 4B).

3.1.3. Effects on the visual and auditory evoked potential

In the VIS evoked responses, there was no consequent effect in the measured parameters. Latency slightly increased till the 30th minute, and after that it was continuously reduced. Duration was higher 10 minutes after the injection of 3-NP, then it was practically equal to the control level in the 30th minute, then again went higher by the 60th minutes, and highest value was measured at the end of the recording (Fig. 5A) – but all these changes were below significance.

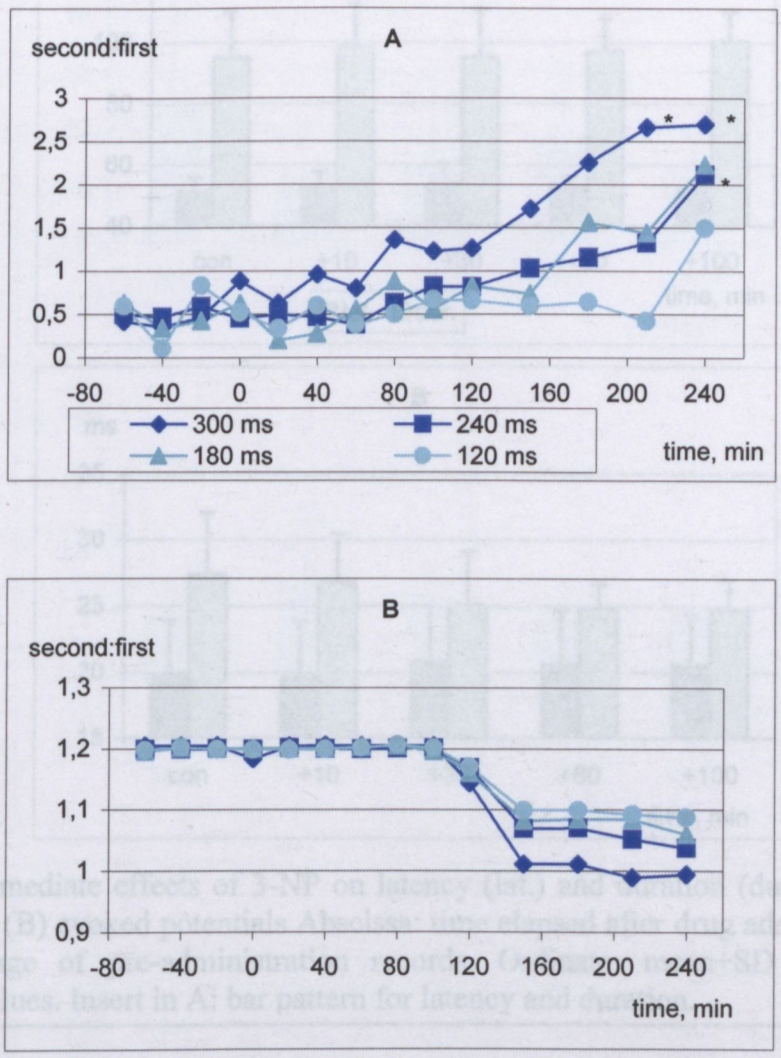


Figure 4 Time course of the amplitude (A) and first peak latency (B) of the SS EPs obtained by double stimuli, in rats before and immediately after application of 3-NP. Abscissa: time (3-NP was given at 0 min). Ordinate: second:first ratio (mean, n=10) of the parameters. *p<0.05 vs. pre-administration period. Insert in A: interstimulus intervals of the double-pulse stimulation.

In the auditory area, the slow-down of the cortical activity following immediate 3-NP administration was paralleled by the increasing latency by the 30th minute and on, and the continuous decreasing in the duration. The shift in both parameter was more pronounced before the 30th minute than after it (Fig. 5B).

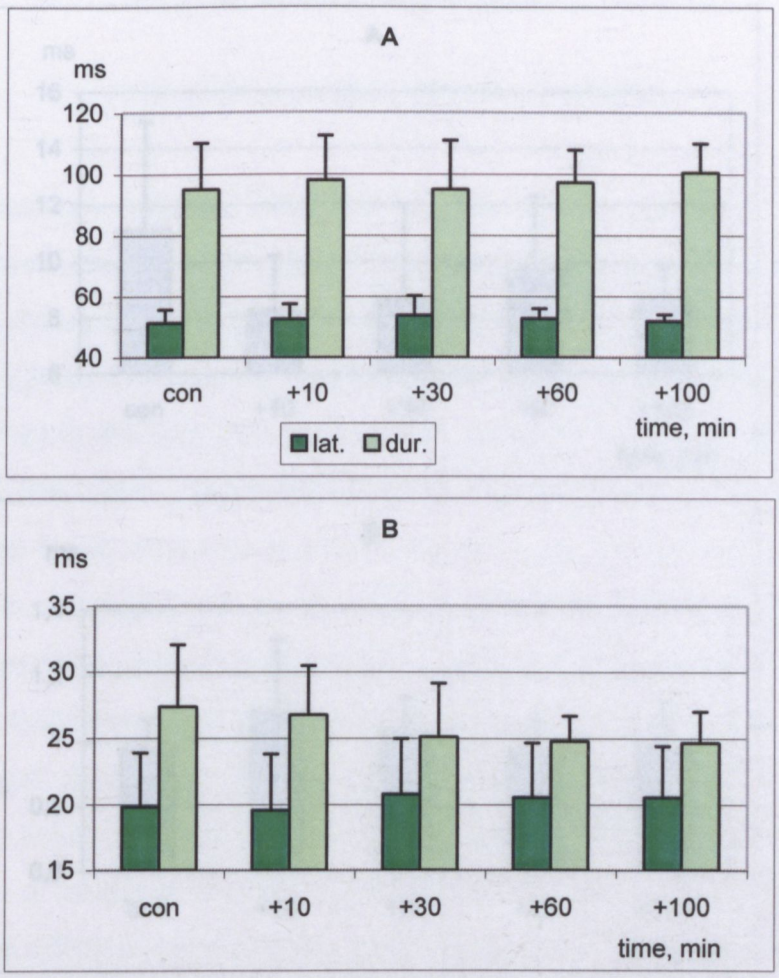


Figure 5 Immediate effects of 3-NP on latency (lat.) and duration (dur.) of visual (A) and auditory (B) evoked potentials Abscissa: time elapsed after drug administration, *con* is the average of pre-administration records. Ordinate: mean+SD (n=10) of the respective values. Insert in A: bar pattern for latency and duration.

3.1.4. Effects of 3-NP on the tail nerve action potential

After immediate application of 20 mg/kg 3-NP, the relative refractory period showed a marked decrease 10 minutes after administration, then it slightly increased to the 60th minute then again decreased, but remained below control level during the whole procedure (Fig. 6A). The absolute refractory period increased slightly till the 10th minute of the experiment, then gradually returned to control level by the 60th minute, and after that it started to increase moderately (Fig. 6B). The value of the conduction velocity was continuously reduced till the 30th minute, then gradually increased surpassing the control level. All of these changes were below significance (Fig. 6C).

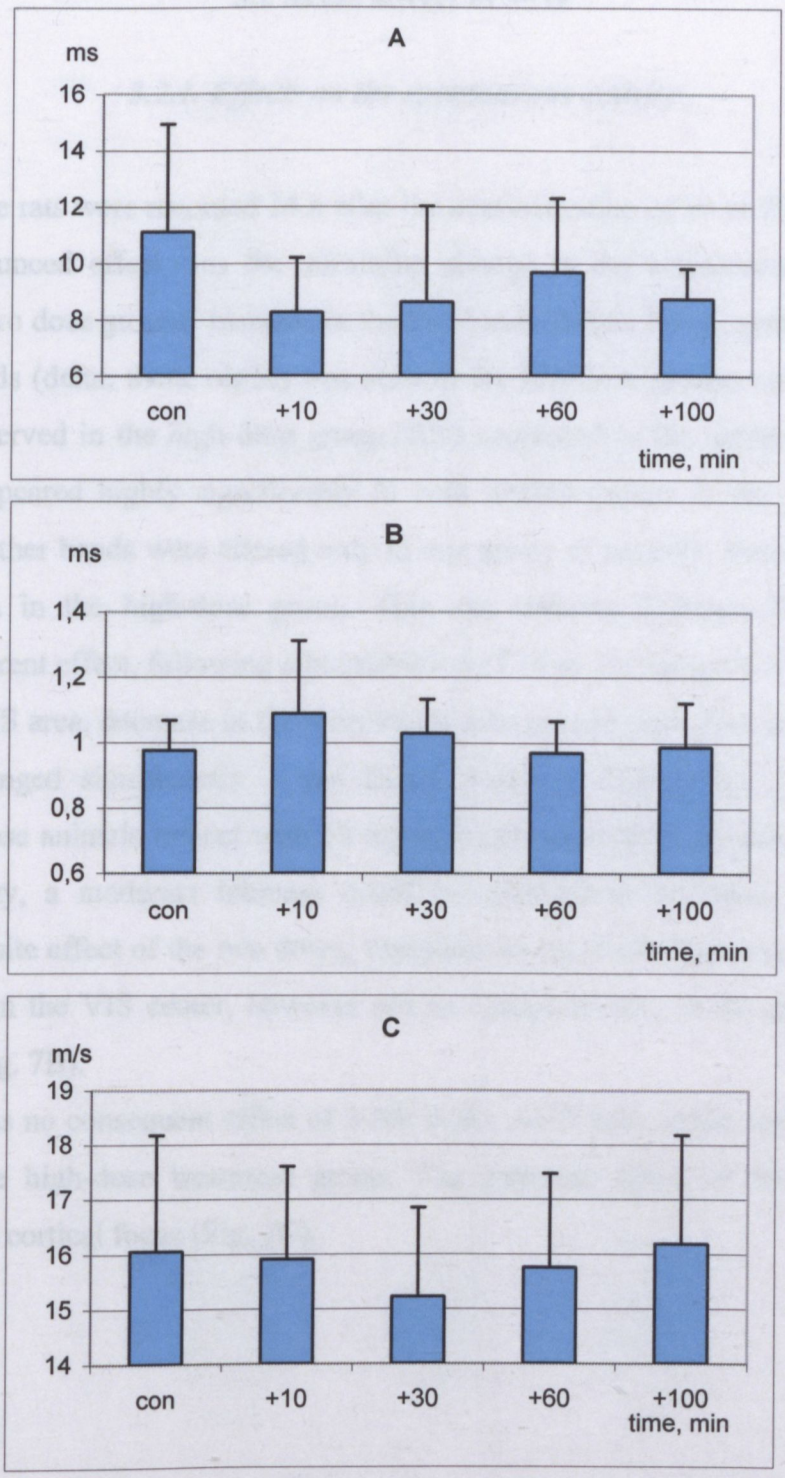


Figure 6 Immediate effects of 3-NP on the relative refractory period time (A), on the absolute refractory period time (B) and on the conduction velocity (C) of the tail nerve. Abscissa: time elapsed after drug administration, *con* is the average of pre-administration records. Ordinate: mean+ SD (n=10) of the respective values.

3.2 Acute effects of 3-NP

3.2.1. Effects on the spontaneous activity

When the rats were recorded 24 h after the administration of 10 or 20 mg/kg 3-NP i.p., the most pronounced effect was the dissimilar change in the somatosensory spontaneous activity in the two dose groups. Increase in the fast bands (beta1, beta2, gamma) and decrease in the slow bands (delta, theta, alpha) was seen in the low-dose group, whereas an opposite change was observed in the high-dose group (both compared to the untreated control). This phenomenon appeared highly significantly in both treated groups in the delta and gamma activity, while other bands were altered only in one group of animals: beta1 in the low-dose, alpha and theta in the high-dose group. This can indicate different time course, or a profoundly different effect, following administration of 10 or 20 mg/kg of 3-NP (Fig. 7A).

In the VIS area, decrease in the slow bands was seen in high-dose animals - theta and alpha band changed significantly – and faster bands of this cortical focus moderately increased. In those animals treated with 10 mg/kg 3-NP, apart from noteworthy reduction of the delta activity, a moderate increase could be detected in all other bands. Thus the apparently opposite effect of the two doses, compared to the control, seen in the SS area, was also detectable in the VIS center, however not so conspicuously, in the gamma, alpha and theta activity (Fig. 7B).

There was no consequent effect of 3-NP in the AUD area, alpha activity significantly decreased in the high-dose treatment group. The converse effect of the two doses was negligible in this cortical focus (Fig. 7C).

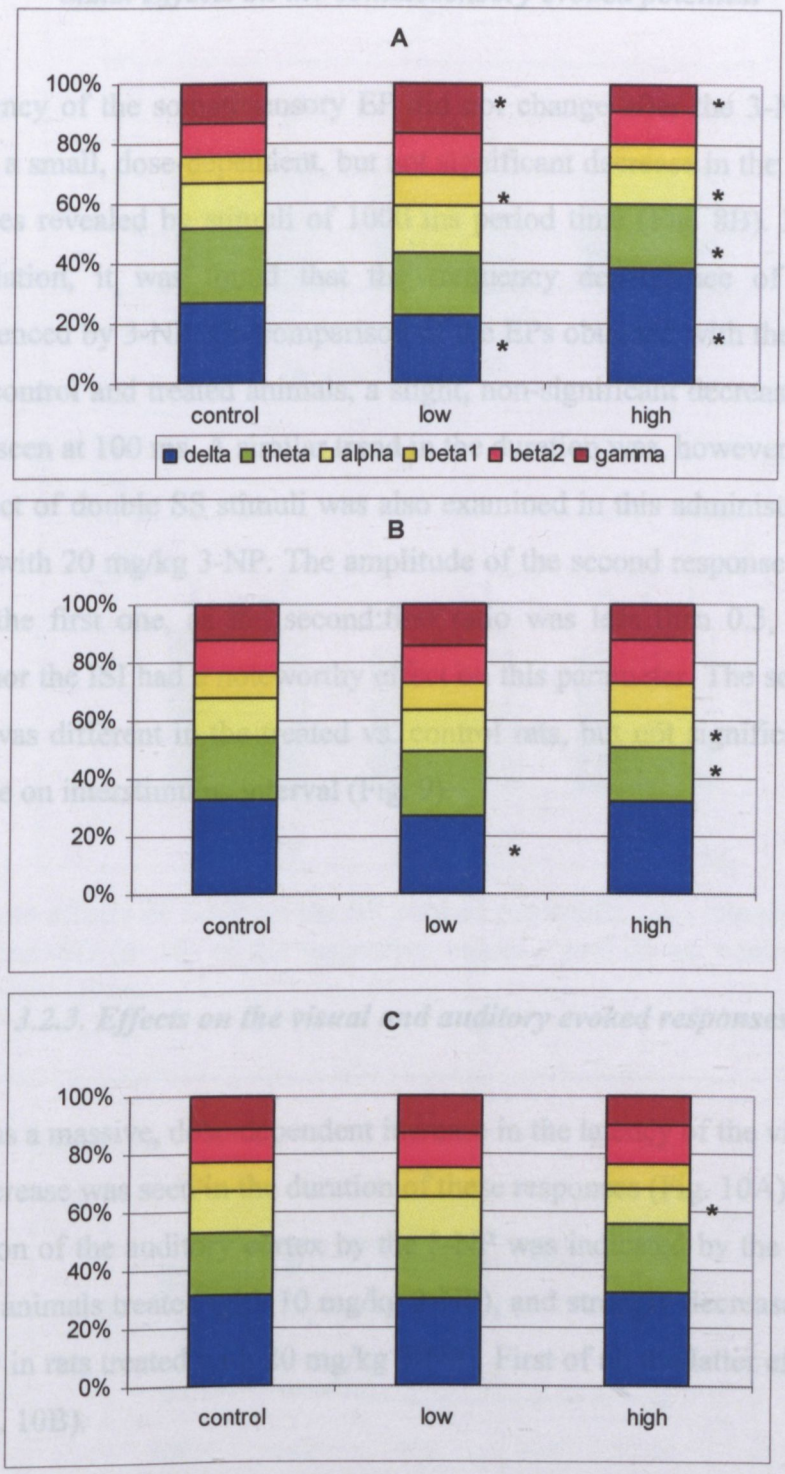


Figure 7 Effects of 3-NP on the spontaneous cortical activity in acute administartion form (A - somatosensory; B - visual; C - auditory area). Ordinate: relative power of the standard frequency bands in control and treated animals (means, n=10) *p<0.05 vs. control in the same band. Insert in A: bar pattern of the frequency bands.

3.2.2. Effects on the somatosensory evoked potential

The latency of the somatosensory EP did not change after the 3-NP treatment (Fig. 8A). There was a small, dose-dependent, but not significant decrease in the duration of the SS evoked responses revealed by stimuli of 1000 ms period time (Fig. 8B). By applying more frequent stimulation, it was found that the frequency dependence of the latency was minimally influenced by 3-NP. On comparison of the EPs obtained with the same stimulation period time in control and treated animals, a slight, non-significant decrease in the high dose vs. control was seen at 100 ms. A similar trend in the duration was, however significant.

The effect of double SS stimuli was also examined in this administration protocol, in the rats treated with 20 mg/kg 3-NP. The amplitude of the second response was considerably suppressed by the first one, as the second:first ratio was less than 0.5, but neither 3-NP administration nor the ISI had a noteworthy effect on this parameter. The second:first ratio of latency values was different in the treated vs. control rats, but not significantly and with no clear dependence on interstimulus interval (Fig. 9).

3.2.3. Effects on the visual and auditory evoked responses

There was a massive, dose-dependent increase in the latency of the visual EP, however only a minor decrease was seen in the duration of these responses (Fig. 10A).

Depression of the auditory cortex by the 3-NP was indicated by the increased latency (significantly in animals treated with 10 mg/kg 3-NP), and strongly decreased duration of the EPs (noteworthy in rats treated with 20 mg/kg 3-NP). First of all the latter effect showed dose dependence (Fig. 10B).

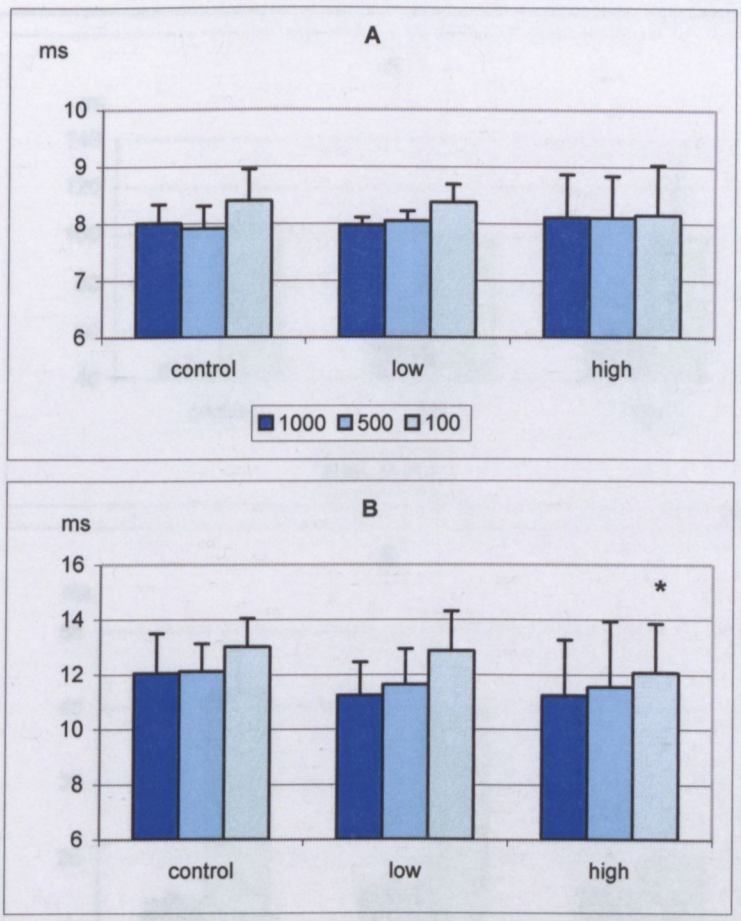


Figure 8 Acute effects of 3-NP on the SS evoked potentials. (A - latency;B - duration). Ordinate: mean+SD (n=10) of the respective values. *p<0.05 vs. control. Insert in A: stimulation period time.

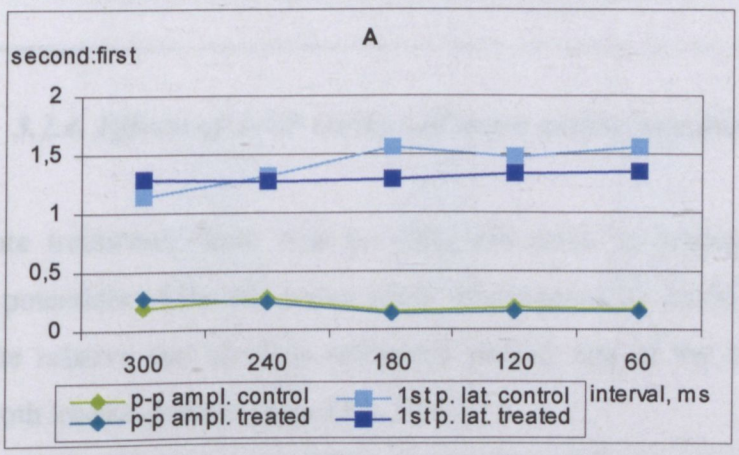


Figure 9 Effect of 3-NP in acutely treated rats on the second:first ratio of amplitude (diamonds) and 1st peak latency (squares) of the SS evoked potential elicited with double stimuli (see Methods). Abscissa: inter-stimulus time. Ordinate: second:first ratio (mean, n=10). Insert: control and treated.

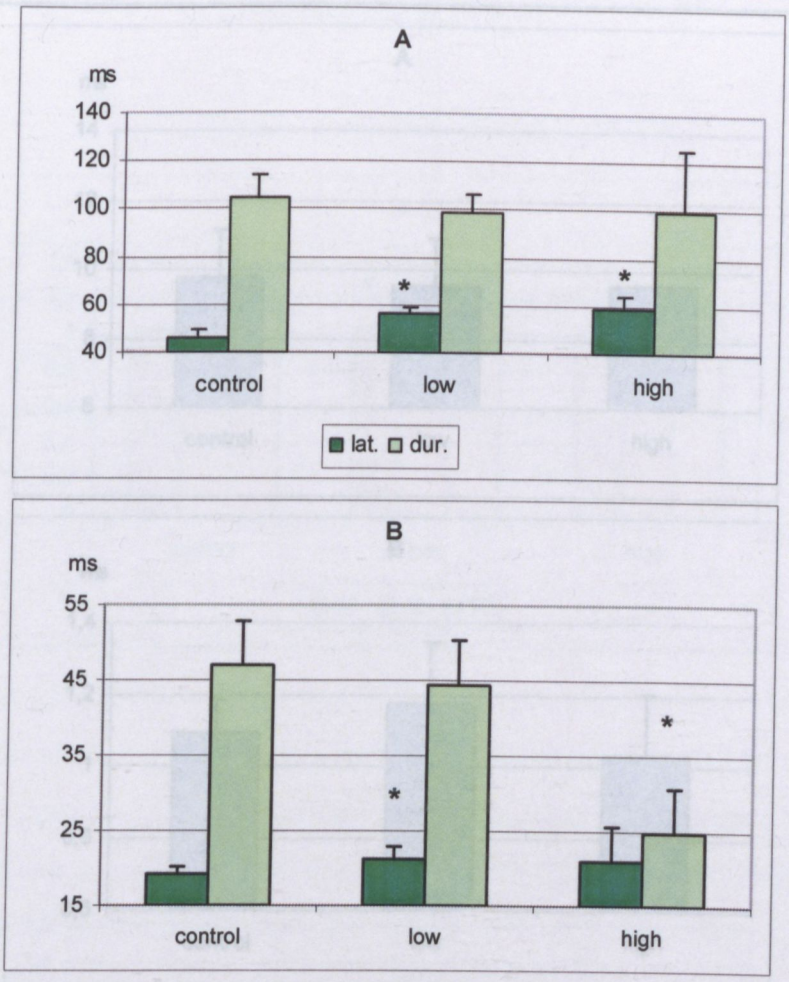


Figure 10 Acute effects of 3-NP on latency (lat.) and duration (dur.) of visual (A) and auditory (B) evoked potentials Ordinate: mean+SD (n=10) of the respective values. *$p<0.05$ vs. control. Insert in A: bar pattern for latency and duration.

3.2.4. Effects of 3-NP on the tail nerve action potential

In the acute treatment, there was no clear alteration in response to 3-NP in the compound action potentials of the tail nerve when investigated by double-pulse stimulation. The changes of the relative and absolute refractory period, and of the conduction velocity, were minimal in both treated groups. (Fig. 11A, B, C).

In rats administered low dose of 3-NP, the latency of the response evoked by 50 ms period time significantly increased. Lower period times (20 or 10 ms) did not affected the this parameter (Fig. 12A). The frequency dependence of the amplitude was practically the same in control and treated animals when varying the period time (Fig. 12B).

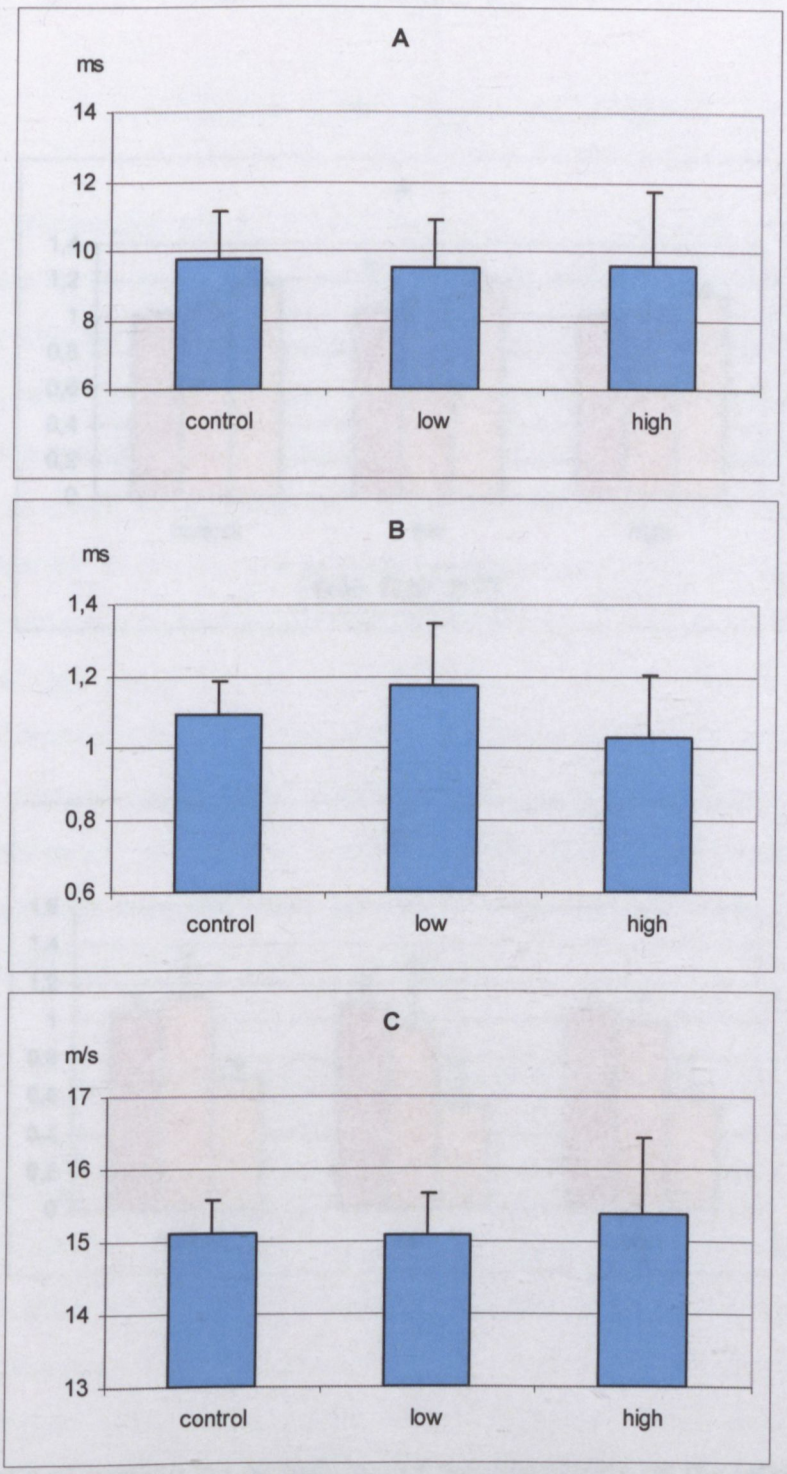


Figure 11 Acute effects of 3-NP on the relative refractory period (A), absolute refractory period (B) and on the conduction velocity (C) of the tail nerve elicited by double pulses. Ordinate: mean+SD, n=10 of the respective values.

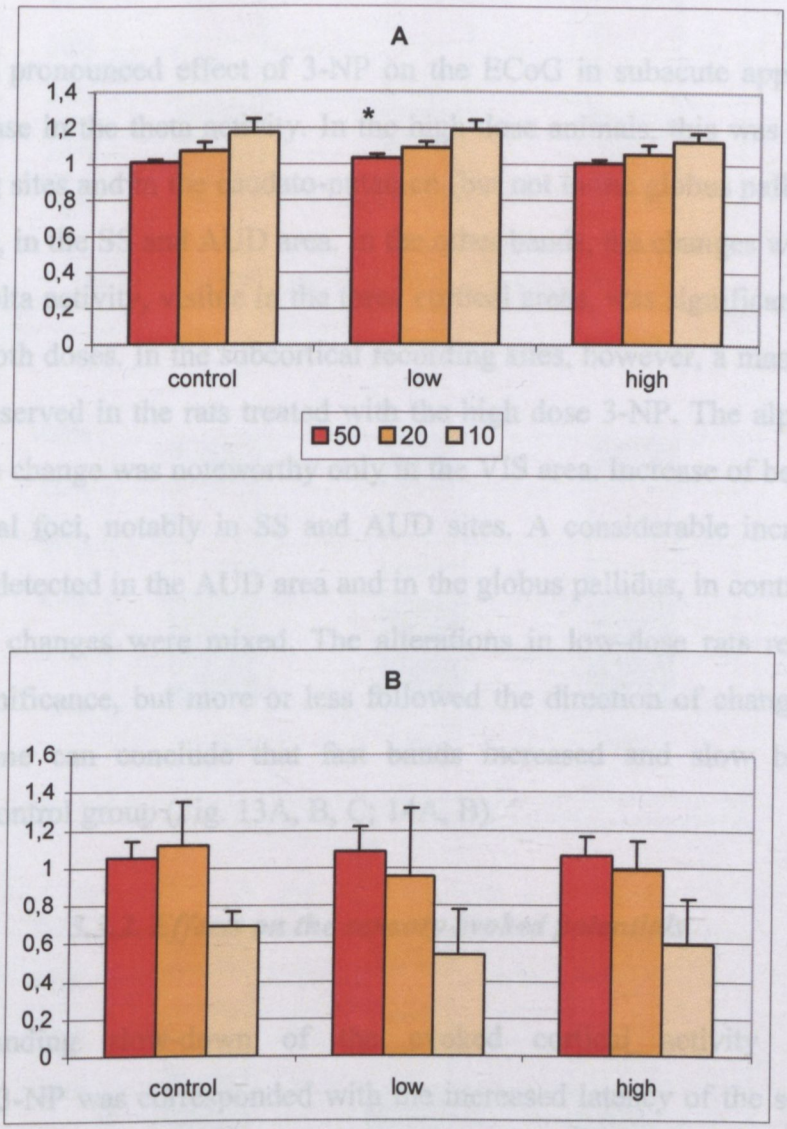


Figure 12 Effects of varying the period time of the stimulation on the latency (A) and amplitude (B) of the tail nerve action potential in rats with acute application of 3-NP. Ordinate: mean+SD, n=10 of the respective values. *p<0.05 vs. control. Insert: stimulus period time.

3.3. Subacute effects of 3-NP

3.3.1. Effects on the spontaneous activity

The most pronounced effect of 3-NP on the ECoG in subacute application was the significant decrease in the theta activity. In the high dose animals, this was seen in all three cortical recording sites and in the caudato-putamen (but not in the globus pallidus), and in the low dose animals, in the SS and AUD area. In the other bands, the changes were not uniform. Increase in the delta activity, visible in the three cortical areas, was significant only in the SS area, following both doses. In the subcortical recording sites, however, a massive decrease of delta could be observed in the rats treated with the high dose 3-NP. The alpha band mainly decreased, but the change was noteworthy only in the VIS area. Increase of beta2 activity was seen in all cortical foci, notably in SS and AUD sites. A considerable increase of gamma activity could be detected in the AUD area and in the globus pallidus, in contrast to any other focus, where the changes were mixed. The alterations in low-dose rats remained, on the whole, below significance, but more or less followed the direction of changes in high-dose rats. Generally one can conclude that fast bands increased and slow bands decreased compared to the control group (Fig. 13A, B, C; 14A, B).

3.3.2. Effects on the sensory evoked potentials

An outstanding slow-down of the evoked cortical activity after subacute administration of 3-NP was corresponded with the increased latency of the sensory EP. The change was significant in all recorded cortical areas and in both treated groups versus control. A slight dose-dependence could be seen, but high vs. low dose differences were only marginal. The alteration in the duration of the evoked responses was moderate (Fig. 15A, B, C). In this administration protocol, the frequency dependence of the EP parameters was not observed.

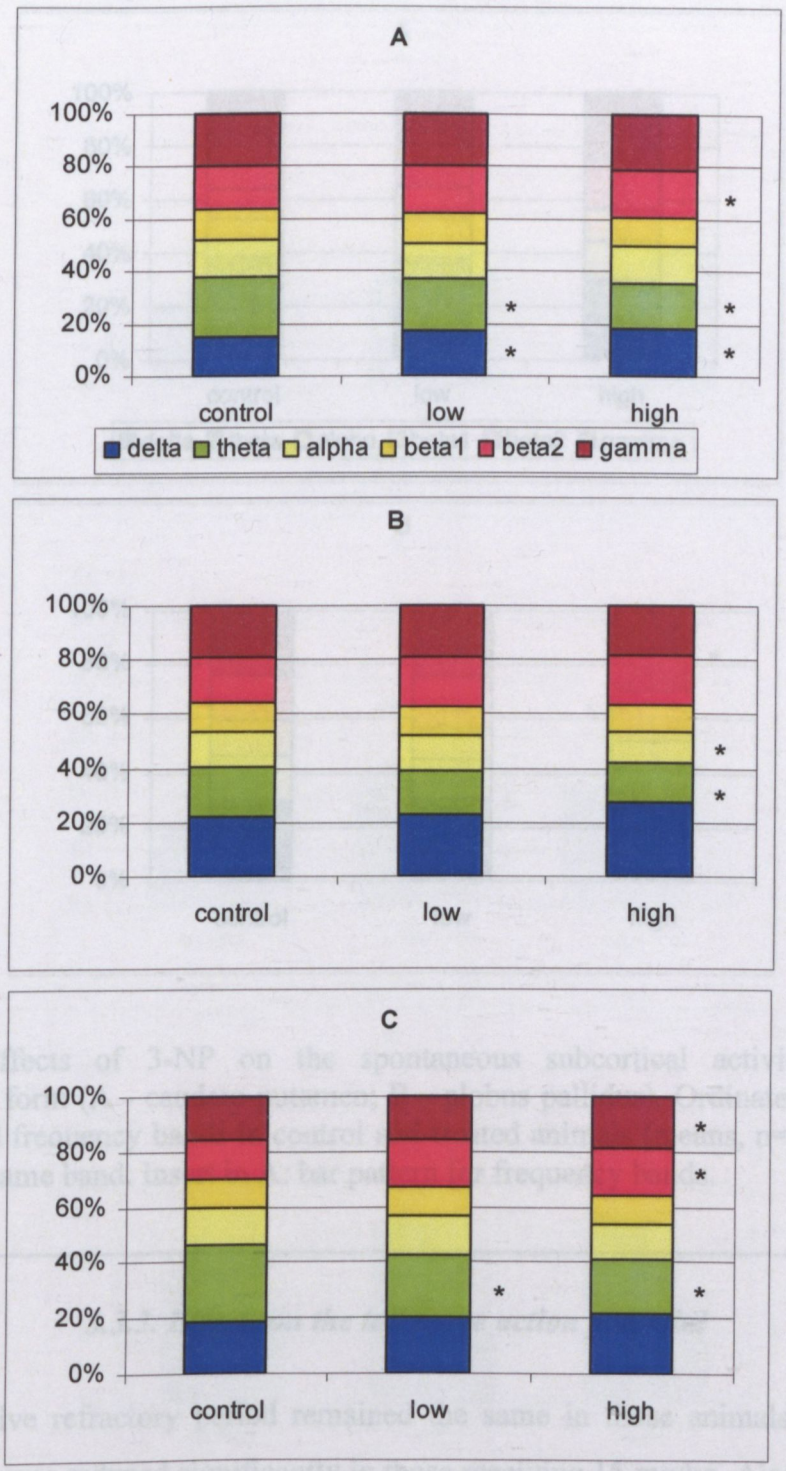


Figure 13 Effect of subacute 3-NP administration on the band spectrum of the spontaneous activity in the three cortical areas (A - somatosensory; B - visual; C - auditory). Abscissa: treatment groups. Ordinate: relative power of the frequency bands (mean, n=10). * $p<0.05$ vs. the same band in the control. Insert in A: bar pattern for frequency bands.

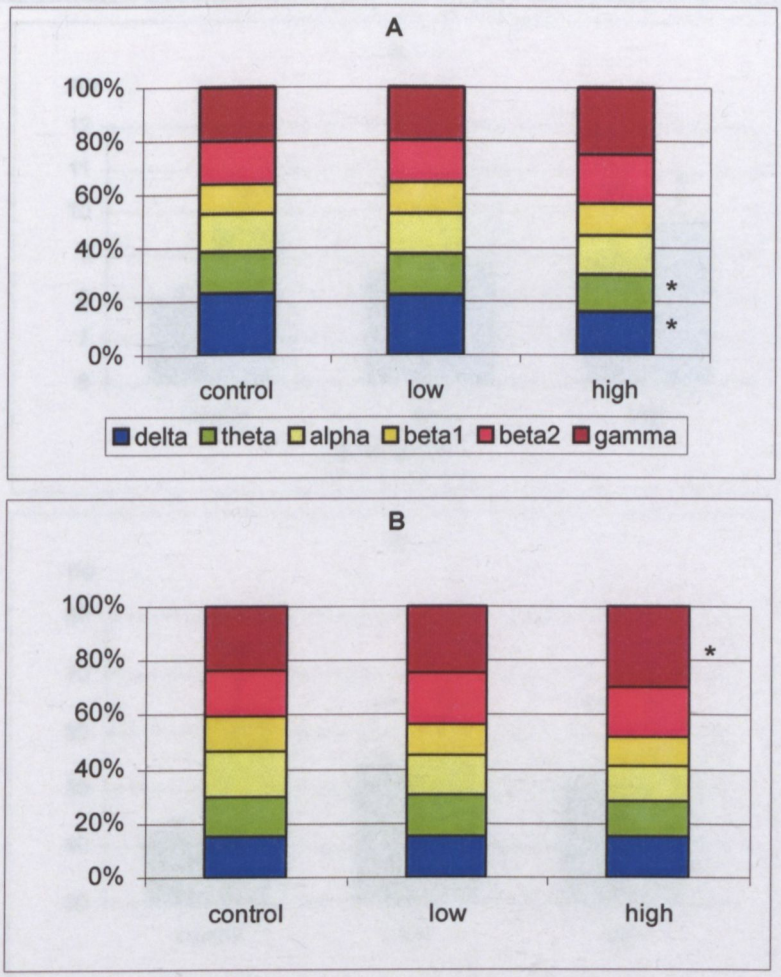


Figure 14 Effects of 3-NP on the spontaneous subcortical activity in subacute administration form (A - caudato-putamen; B - globus pallidus). Ordinate: relative power of the standard frequency bands in control and treated animals (means, n=10) *p<0.05 vs. control in the same band. Insert in A: bar pattern for frequency bands.

3.3.3. Effects on the tail nerve action potential

The relative refractory period remained the same in those animals treated with 10 mg/kg 3-NP, but was reduced significantly in those receiving 15 mg/kg. Also a converse shift of the two doses was seen in alteration of the absolute refractory period: this parameter was reduced at low-dose administered animals, but in the other group it increased notably compared to the control group. Conduction velocity showed the most conspicouos change from the double-pulse evoked tail nerve action potentials, an evident, highly significant increase happened after application of both doses (Fig. 16A, B, C).

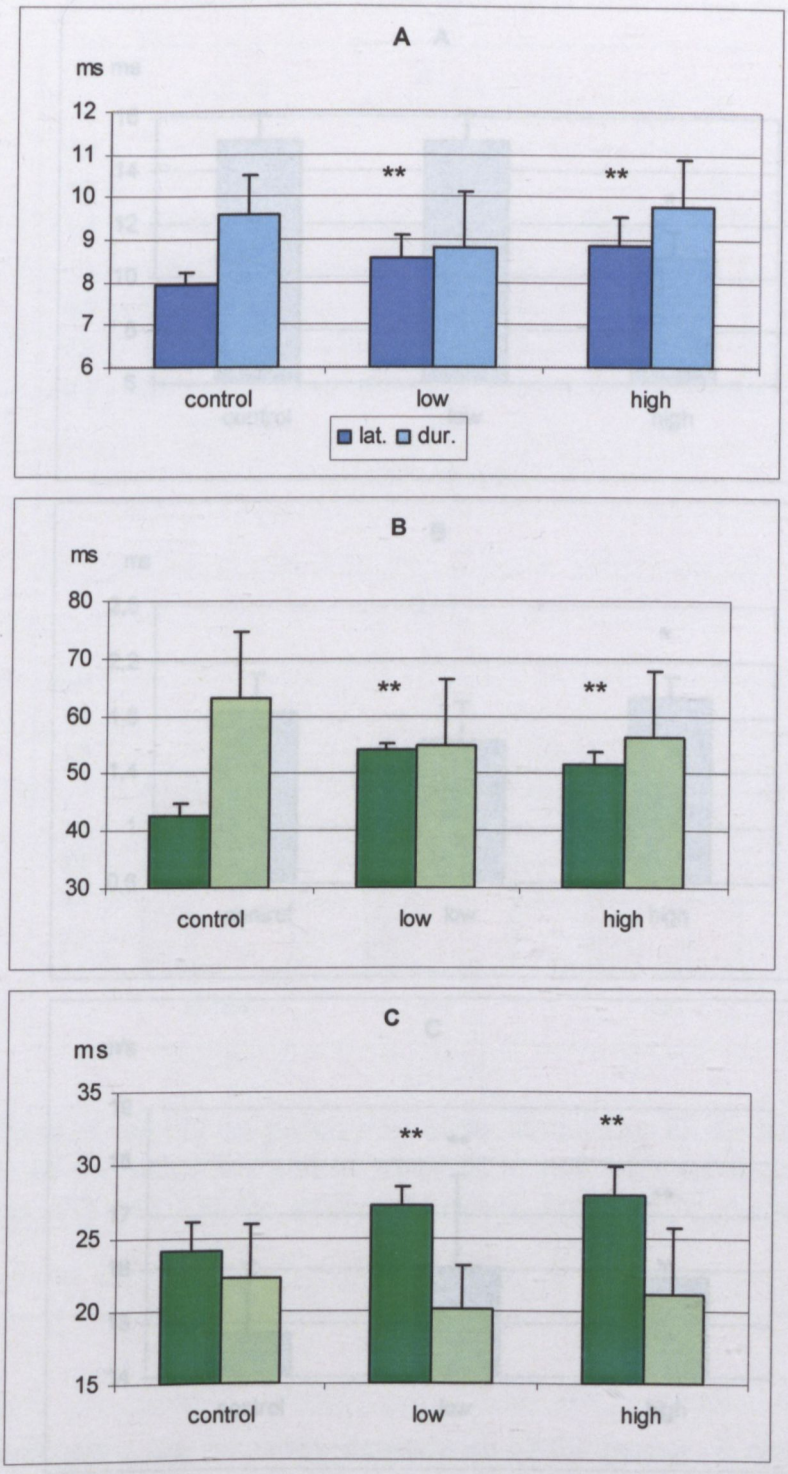


Figure 15 Subacute effects of 3-NP on latency (lat.) and duration (dur.) of somatosensory (A), visual (B) and auditory (C) evoked potentials Ordinate: mean+SD (n=10) of the respective values. $^{**}p<0.01$ vs. control. Insert in A: bar pattern for latency and duration.

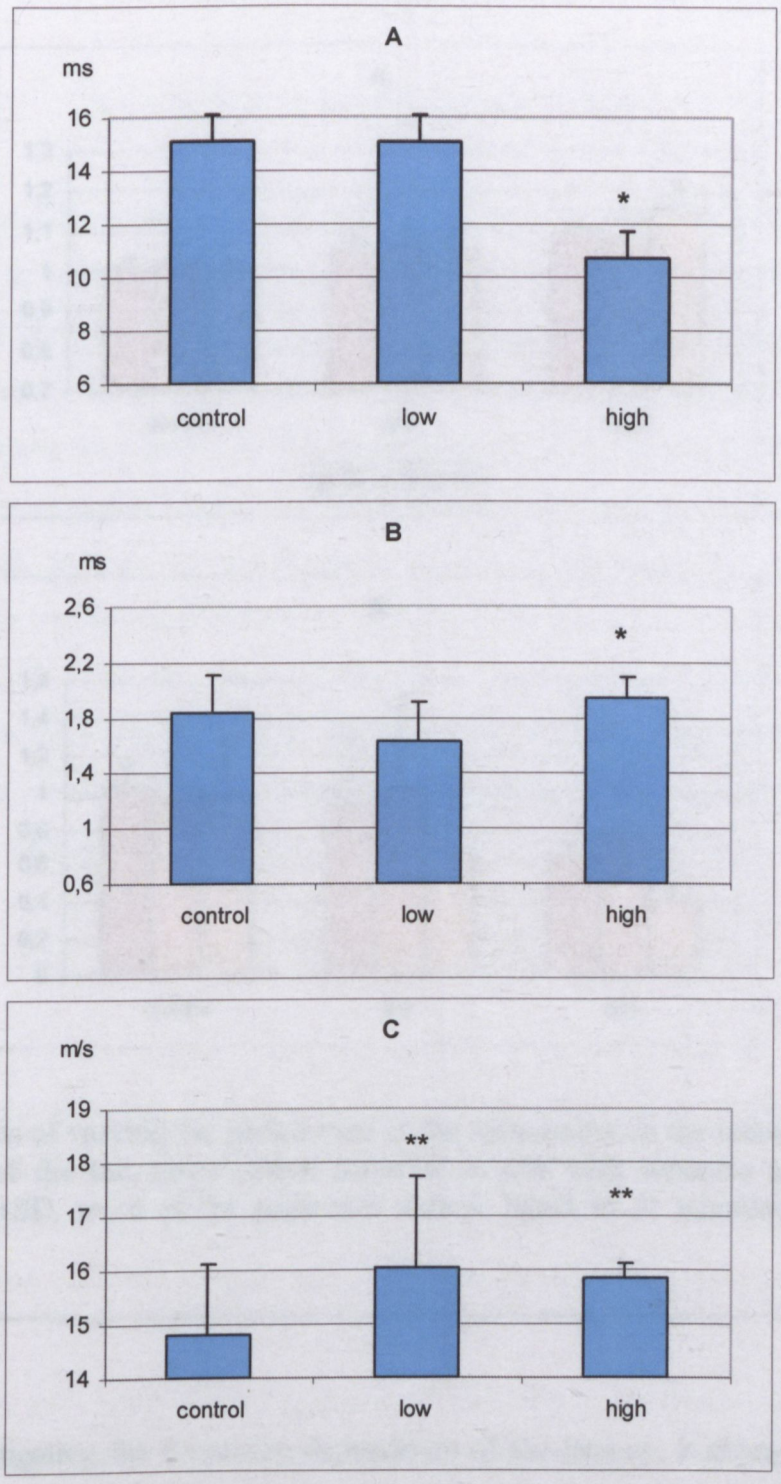


Figure 16 Subacute effects of 3-NP on the relative refractory period (A), absolute refractory period (B) and on the conduction velocity (C) of the tail nerve elicited by double pulses. Ordinate: mean+SD, n=10 of the respective values. *p<0.05, **p<0.01 vs. control.

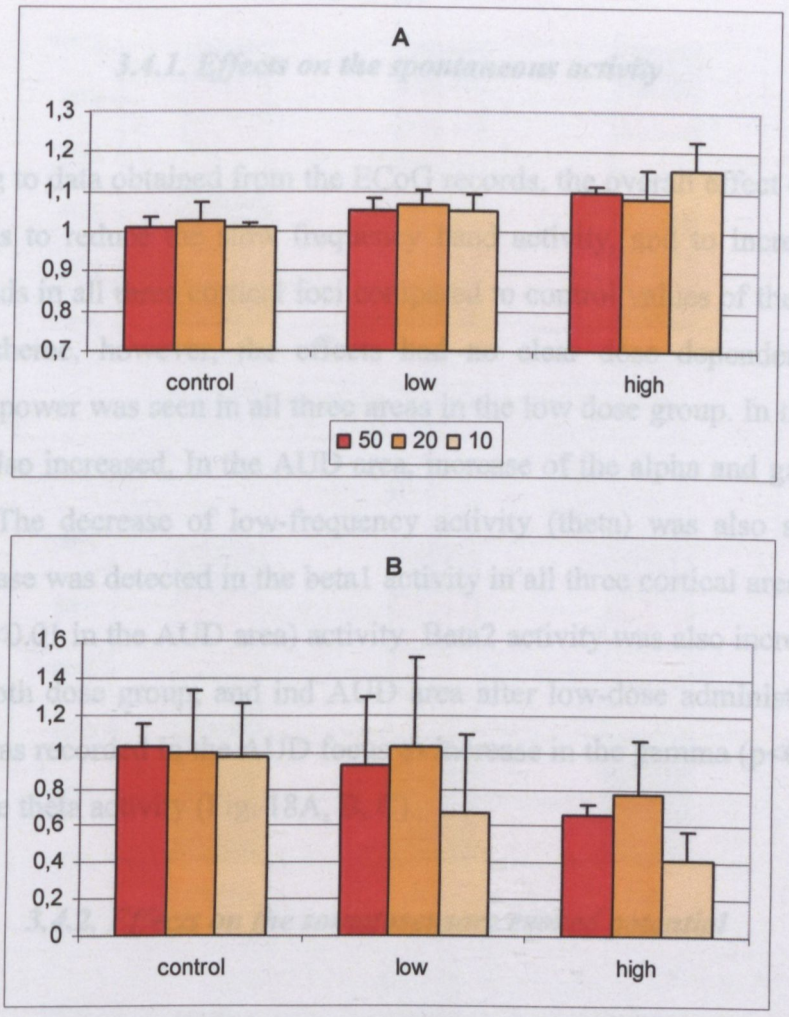


Figure 17 Effects of varying the period time of the stimulation on the latency (A) and amplitude (B) of the tail nerve action potential in rats with subacute application. Ordinate: mean+SD, n=10 of the respective values. Insert in A: stimulation period time.

When investigating the frequency dependence of the latency, it changed moderately but indicated the impairment of the excitation mechanism by 3-NP, because it lengthened in the treated animals compared to the paralell controls (Fig. 17A). The same deterioration of excitation mechanism was seen in the amplitude, but the frequency dependence was more marked, although not significantly influenced by 3-NP.

3.4. Subchronic effects of 3-NP

3.4.1. Effects on the spontaneous activity

According to data obtained from the ECoG records, the overall effect of subchronic 3-NP treatment was to reduce the slow frequency band activity, and to increase the relative power of fast bands in all three cortical foci compared to control values of the same bands. In this treatment scheme, however, the effects had no clear dose dependence. Significant increase of beta1 power was seen in all three areas in the low dose group. In the SS and AUD area, beta2 was also increased. In the AUD area, increase of the alpha and gamma band was also significant. The decrease of low-frequency activity (theta) was also significant here. Noteworthy increase was detected in the beta1 activity in all three cortical area after low-dose administration ($p<0.01$ in the AUD area) activity. Beta2 activity was also increased notably in the SS area, in both dose group; and ind AUD area after low-dose administration. Another significant shift was recorded in the AUD focus as increase in the gamma ($p<0.01$) and alpha, and decrease in the theta activity (Fig. 18A, B, C).

3.4.2. Effects on the somatosensory evoked potential

In accordance with the alteration of the spontaneous activity, increase of cortical activity was experienced as latency decrease was measured dose-dependently, but not notably, when SS EPs were examined (Fig. 19A). Duration of the elicited SS responses became higher after 3-NP application, and this increase was significant in high-dose group of animals (Fig. 19B).

Investigating the frequency dependence of SS EPs, a moderate, dose-dependent reduction of the latency could be seen, but without significance, even if the period time of the stimuli was shortened (500 and 100 ms; Fig. 19A). An opposite shift could be seen in frequency dependence of the duration, the time of this parameter extended, in higher compass in the low-dose group, moreover 500 ms period time evoked response significantly altered (Fig. 19B).

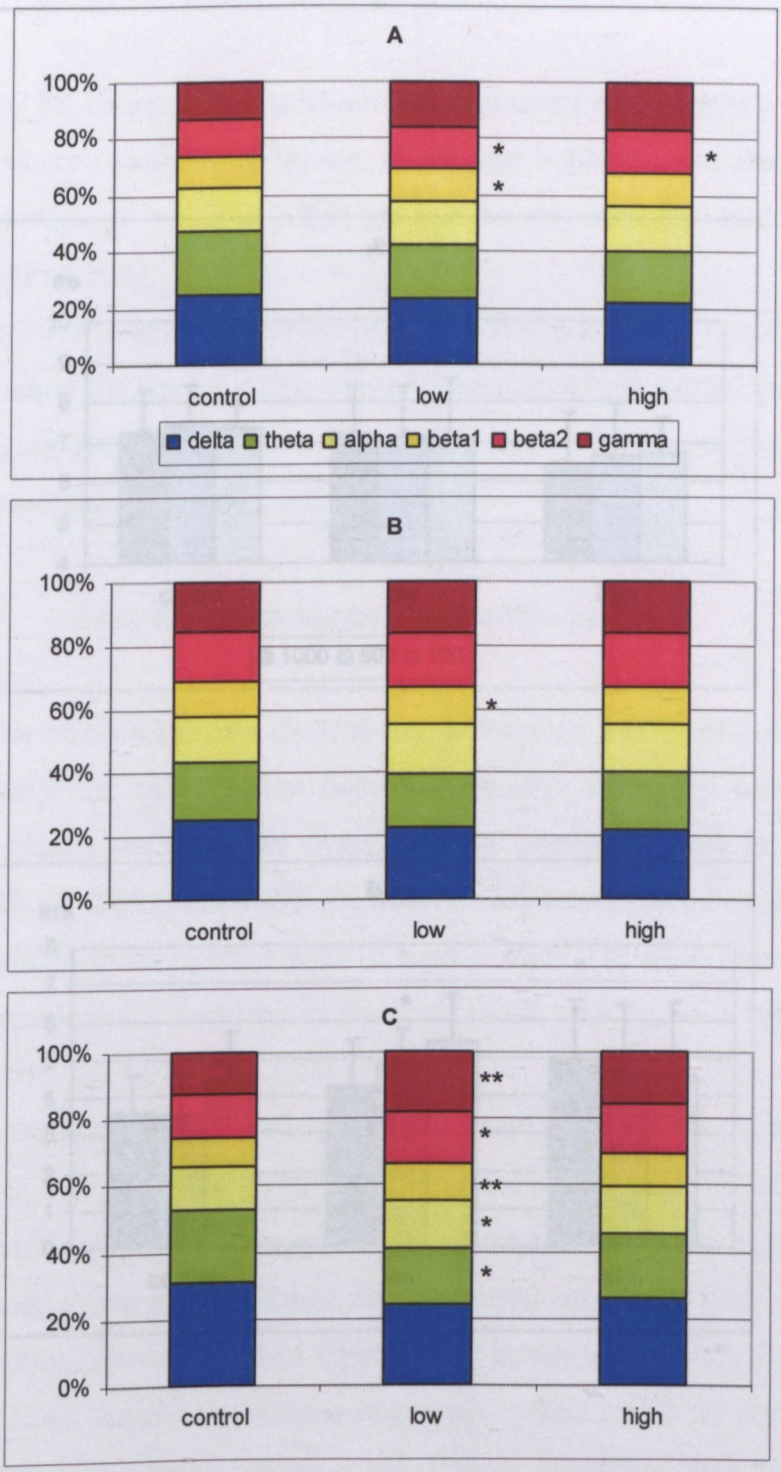
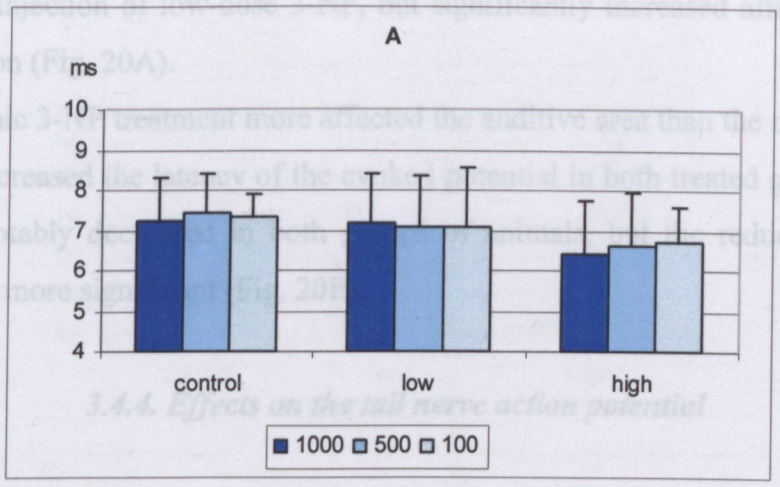


Figure 18. Effect of subchronic 3-NP administration on the band spectrum of the spontaneous activity in the three cortical areas (A - somatosensory; B - visual; C - auditory). Abscissa: treatment groups. Ordinate: relative power of the frequency bands (mean, n=10). * $p<0.05$ ** $p=0.01$ vs. the same band in the control. Insert in A: bar pattern for frequency bands.



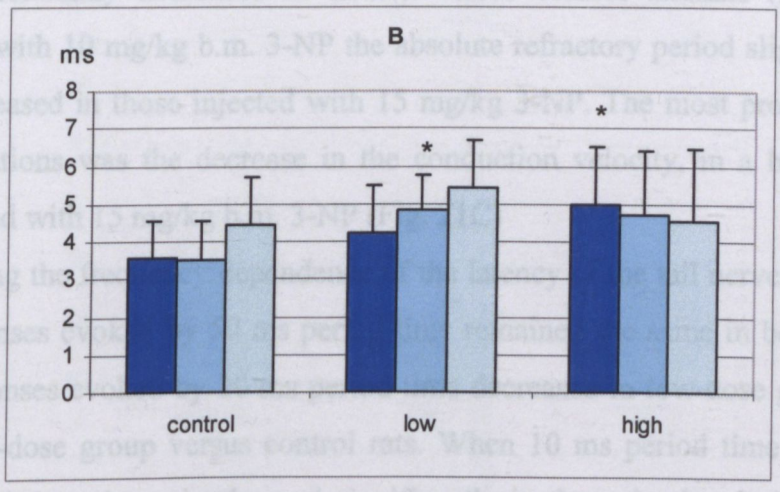


Figure 19 Subchronic effects of 3-NP on the somatosensory evoked potentials with different period times (1000, 500 and 100 ms). (A - latency; B - duration). Ordinate: mean+SD, n=10 of the respective values *$p<0.05$. Insert in A: stimulation period time.

3.4.3. Effects on the visual and auditory evoked responses

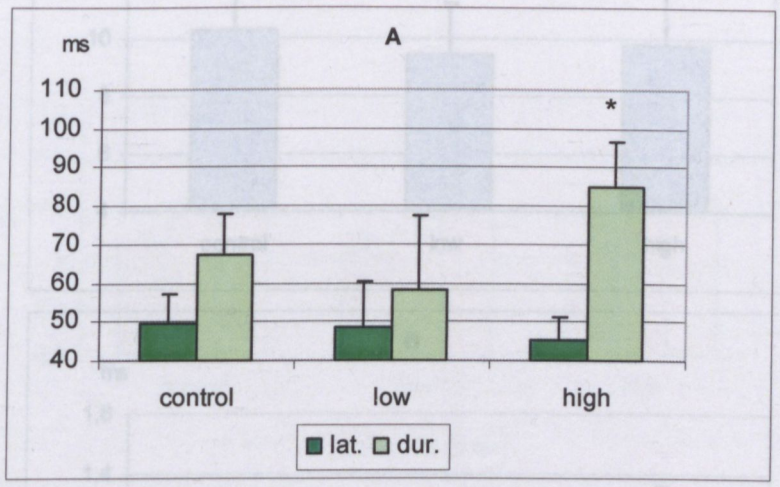
Similarly to SS changes, the same alteration occured in parameters of responses by VIS stimulation, where reduction of latency values was observed, and duration marginally decreased after injection of low-dose 3-NP, but significantly increased after high-dose of 3-NP administration (Fig. 20A).

Subchronic 3-NP treatment more affected the auditive area than the other two, because noteworthyly decreased the latency of the evoked potential in both treated group. Duration of the responses notably decreased in both groups of animals, but the reduction in low-dose group was much more significant (Fig. 20B).

3.4.4. Effects on the tail nerve action potential

Double-pulse stimulation data showed that subchronic 3-NP treatment did not affect noteworthy the peripheral nerve action potential. Relative refractory period slightly and inverse-dose-dependently decreased in treated versus control animals (Fig. 21A, B). In animals treated with 10 mg/kg b.m. 3-NP the absolute refractory period slightly reduced, but moderately increased in those injected with 15 mg/kg 3-NP. The most pronounced effect in peripheral alterations was the decrease in the conduction velocity, in a higher compass in those rats injected with 15 mg/kg b.m. 3-NP (Fig. 21C)

Examining the frequency dependence of the latency of the tail nerve action potentials, latency of responses evoked by 50 ms period time remained the same in both treated group. Latency of responses evoked by 20 ms period time decreased in low-dose group much more then in the high-dose group versus control rats. When 10 ms period time was applied, the latency decreased dose-dependently, and significantly in the animals administered with 15 mg/kg 3-NP (Fig. 22A). Amplitude of those responses evoked by 50 ms period time did not changed at all compared to paralell control group. During stimulation with 20 ms period time the amplitude extended gradually inversely to increasing doses (Fig. 22B). Unfortunatelly, the amplitude of those responses elicited by 10 ms period time were so low that we were technically unable to measure it.



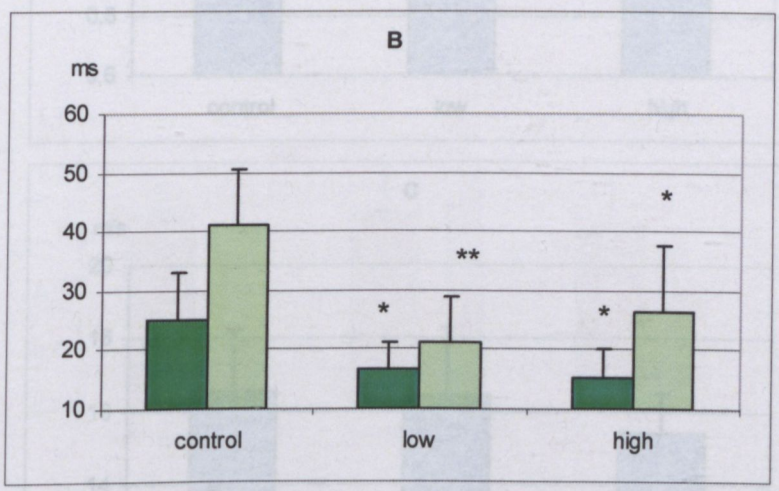


Figure 20 Subchronic effects of 3-NP on latency (lat.) and duration (dur.) of visual (A) and auditory (B) evoked potentials. Ordinate: mean+SD n=10 of the respective values. *$p<0.05$, **$p<0.01$ vs. control. Insert in A: bar pattern for latency and duration.

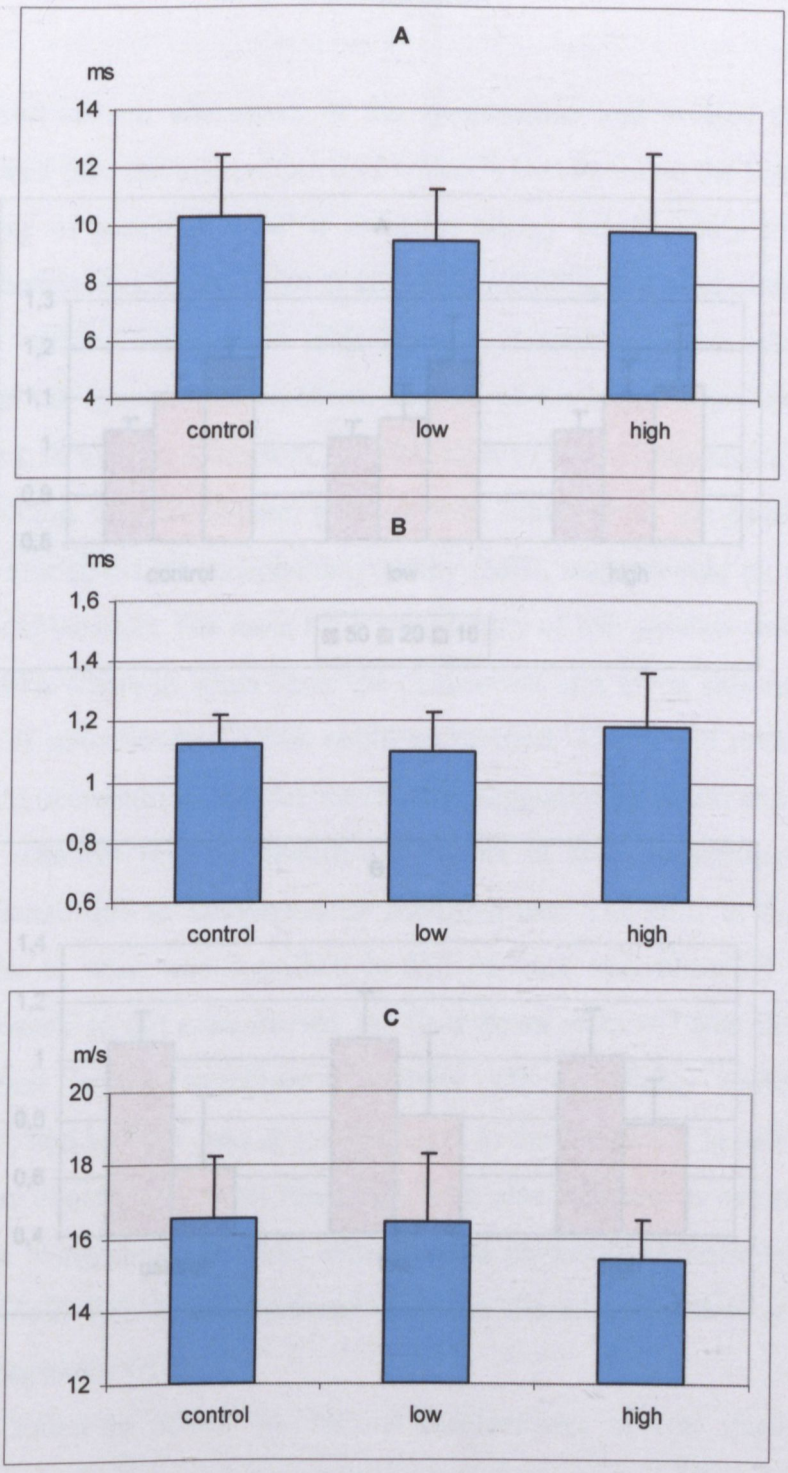


Figure 21 Subchronic effects of 3-NP on the relative refractory period (A), absolute refractory period (B) and on the conduction velocity (C) of the tail nerve elicited by double pulses. Ordinate: mean+SD, n=10 of the respective values.

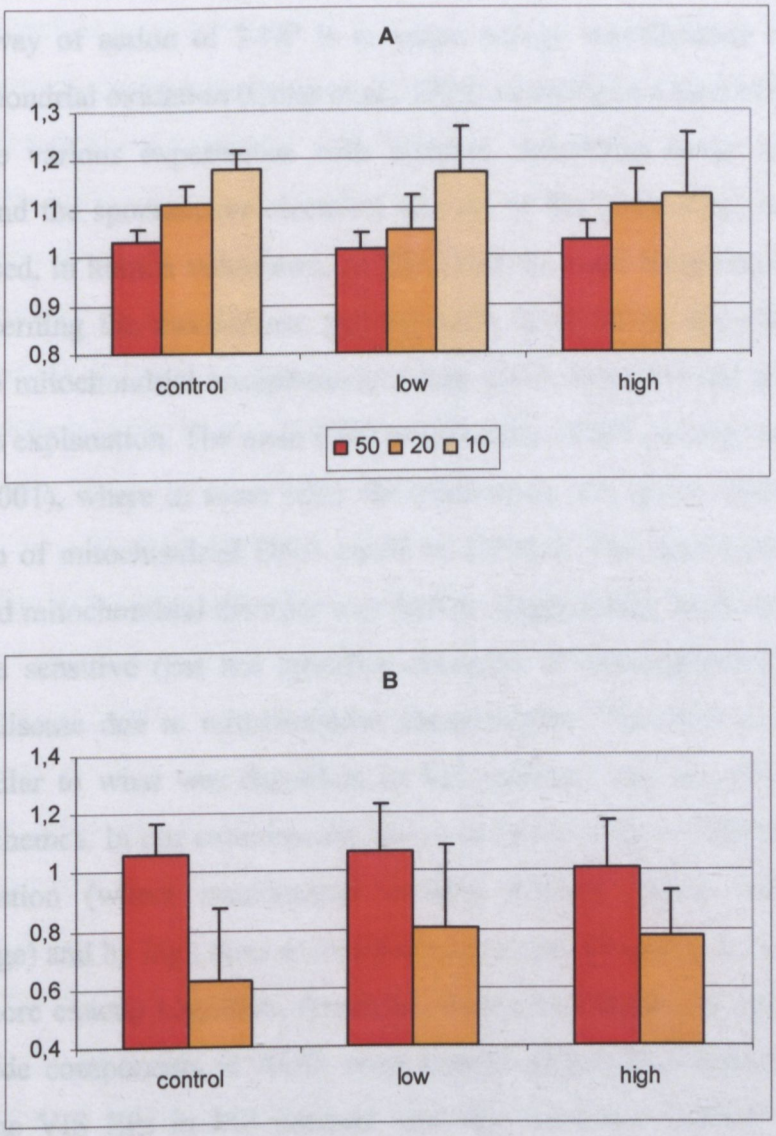


Figure 22 Effects of varying the period time of the stimulation on the latency (A) and amplitude (B) of the tail nerve action potential in rats with subchronic application. Ordinate: mean+SD, n=10 of the respective values. Insert in A: stimulation period time.

4. Discussion

3-NP caused several alterations in the spontaneous and evoked cortical electrical activity of the treated rats, the interpretation of which is best based on the known actions of 3-NP. The main way of action of 3-NP is to cause energy insufficiency in the neurons by inhibiting mitochondrial oxidation (Coles et al., 1979) resulting in a kind of tissue hypoxia.

There are various experiences with humans, describing some relationship of the energetic state and the spontaneous electrical activity of the brain. Exposure to low oxygen gas mixture caused, in human volunteers, an EEG shift to lower frequencies (van der Post et al., 2002). Concerning the mechanism, patients with inherited or idiopathic mitochondrial dysfunction, like mitochondrial encephalomyopathy (ME), may provide an even better base for a mechanistic explanation. The main EEG abnormality of ME patients was slowed activity (Sciacco et al, 2001), where in some cases the connection of a given abnormal EEG pattern and the mutation of mitochondrial DNA could be verified. The causal relationship of EEG abnormalities and mitochondrial disorder was further supported by Smith and Harding (1993) who stressed the sensitive (but not specific) character of electrophysiological methods in detecting CNS disease due to mitochondrial abnormalities. The shift in the ECoG to low frequencies, similar to what was described in ME patients, was observed in our study in certain dosing schemes. In our experiments, the slow-down of ECoG was clear in immediate 3-NP administration (where spontaneous activity showed highly uniform, albeit not significant, change) and by high dose acute treatment, in the SS cortex. In ME patients, higher order cortical, more exactly cognitive, functions, were also affected, as evidenced by slowed and low-amplitude components in AUD event-related potentials (Montirosso et al., 2002). Alterations of the VIS EPs in ME patients was also described (Scaioli et al., 1998) and claimed to be of diagnostic value.

3-NP was found to affect, by various mechanisms, several transmitter systems, glutamatergic transmission being the most studied in this aspect. 3-NP is known to inhibit glutamate uptake (Tavares et al., 2001). Beside contributing to excitotoxic neuronal loss (Pubill et al., 2001), this probably leads to imbalance between excitation and inhibition. The resulting shortage of transmitter in the presynaptic ending may explain the significant decrease of somatosensory EP duration obtained by frequent stimulation 24 h after 20 mg/kg

3-NP administration. Over a longer period, abnormally high levels of glutamate in the interneuronal space cause likely a desensitization of the receptors, expressed in the latency lengthening of the EP, seen in all three modalities in the subacute treatment protocol. Inhibition of glutamate uptake may influence spontaneous cortical activity indirectly, via the ascending cholinergic activation (Metherate et al., 1992). The glutamate receptors on the cholinergic neurons in the basal forebrain (Fournier et al., 2004) are first over-excited, then desensitized, with parallel alterations of the ascending cortical activation.

The results obtained by paired-pulse SS stimulation provide further support to the adequacy of a disease model based on 3-NP and electrophysiological measurements. The change of the second:first ratio of the EP amplitudes following 3-NP administration reflect probably a kind of disinhibition. In human ME patients, paired-pulse SS stimulation delivered to the median nerve revealed strongly reduced intracortical inhibition (Liepert et al., 2001). Although this was a phenomenon observed in humans and the ISIs were not comparable to those used by us, the similarity of the effects, further the known connection of mitochondriopathy with increased cortical excitability (Rosing et al., 1985) and altered γ-aminobutyric acid (GABA) levels (Erecinska and Nelson, 1994), suggest that double-pulse stimulation can be a sensitive and specific means to reveal the action of 3-NP in the CNS. In the acute protocol, no noteworthy effect on the second:first ratio was seen any more, indicating that the effect was probably directly caused by energy insufficiency (lack of ATP) which is constantly present in ME patients but gradually developed and declined in the treated rats in the course of 24 hours.

One of the striking findings in the present work was that the effect of 3-NP administration on the spectrum of ECoG was opposite in different treatment protocols. This may have been due to a complex dose- and time-dependence of 3-NP effects. In the two treatment protocols designated subacute and subchronic in this study, for example, the direction of change of the ECoG parameters was opposite. This, however, showed a good parallelism with the changes of motor behaviour described in the literature. By applying the same treatment scheme as our subchronic one (one ip. injection of 10 mg/kg 3-NP every 4th day: Borlongan et al., 1997a) motor hyperactivity was observed up to the 4th injection, and hypoactivity from then on, and the cortical electrical activity showed an opposite pattern. In the rats treated this way, the ECoG was shifted to higher frequencies in our work, whereas the

subacute treatment with altogether 5 doses in a shorter period of time, which caused slowed ECoG, may better correspond to the first phase of the treatment course of Borlongan et al. The importance of timing in 3-NP administration to rats in modelling HD was also stressed by Guyot et al., 1997. There, subacute daily injections and chronic continuous infusion resulted in dissimilar pathology of the striatum and motor abnormalities of rats, and the outcome of the chronic administration was found to be the more adequate model of HD.

A further factor influencing the effect of 3-NP administration is the dose. The ECoG change obtained by injection of 20 mg/kg 3-NP was similar in the immediate and acute treatment protocol. The low dose (10 mg/kg) had here no effect (or caused a slight, by far non-significant acceleration). In other – histological and behavioural – outcomes. a similar dose dependence was mentioned by Kodsi and Swerdlow (1997).

In case of disease models based on a toxic effect in the broad sense, it is an important question whether the alterations regarded as an analogue of the human disease in question are caused directly or are secondary consequences of an effect of the drug which is outside the scope of the disease to be modelled. As the mitochondrial effect of 3-NP is not restricted to the brain (Alexi et al., 2000) the changes of the cortical electrical activity may have resulted from systemic mitochondrial damage. This damage, of course, cannot be fully disregarded - but the fact that in our study no alteration in the body and organ weights were seen in the rats treated by the subacute and subchronic protocol on comparison with the untreated controls, indicated that such a secondary effect was unlikely. Evaluation of the alterations in the tail nerve parameters from this aspect showed that these were not in line with those of the cortical EPs, which again supports that the latter indicated an effect of 3-NP on the cortical activity itself.

So far, there have been hardly any data in the literature about functional neurotoxicological alterations caused by 3-NP, as mainly the histological aspects of HD, as well as alterations in the motor behaviour, were investigated in the animal models developed. The results of the present study and their evaluation showed that a functional approach, based on electrophysiological techniques, can be useful in detection and follow-up of the CNS effects of 3-NP (and possibly other agents used in modelling HD and other chronic degenerative diseases of the human brain) and can point to new questions. According to the first point of the aims set forth (see 1.5.) it can be concluded that the electrophysiological

methodology established at the Department was suitable for detecting the functional changes caused by 3-NP. Further, the standard methods could be developed, primarily with the technique of repeated sensory stimulation, to accommodate it to the specific character of the alterations caused by 3-NP. The question, which of the parameters recorded and analysed is, based on sensitivity and specificity, best suitable to follow-up the development of damage caused by 3-NP, cannot be definitely answered at the moment. Supposing that the necessary chronic recording technique is available, SS stimulation with varied pulse pattern (double, slow-fast) seems promising. Another important question to be solved in the future is to what extent the functional changes indicated by the electrophysiolgical parameters and the histological or biochemical changes run in parallel. Answering these questions would provide another research tool, applicable in basic research and pharmacological development in the field of HD.

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7. Appendix

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